

# **Alcohol and Opiates**

NEUROCHEMICAL AND BEHAVIORAL  
MECHANISMS

Edited by

**Kenneth Blum**

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# Alcohol and Opiates

## NEUROCHEMICAL AND BEHAVIORAL MECHANISMS

Edited by

**Kenneth Blum**

*Chief, Division of Drug and Alcohol Abuse  
and*

*Associate Professor  
Department of Pharmacology  
University of Texas  
Health Science Center  
San Antonio, Texas*

*Associate Editors*

**Diana L. Bard and Murray G. Hamilton**

*Division of Drug and Alcohol Abuse  
Department of Pharmacology  
University of Texas  
Health Science Center  
San Antonio, Texas*



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This book is dedicated to my wife, Arlene Carol, for her unselfish love and magnificent devotion; and to our children, Jeffrey Harris and Seth Howard, for whom we wish a life of pleasure inducement that transcends the hangups and misconceptions of our contemporary adult world.

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# List of Contributors

- L. G. ABOOD, Center Brain Research and Biochemistry, University of Rochester Medical Center, Rochester, New York 14642
- KENNETH BLUM, Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284
- HOWARD CAPPELL, Research Division, Addiction Research Foundation, 33 Russell Street, Toronto, Ontario, Canada M5S 2S1
- H. LEE CARDENAS, Departments of Pharmacology and Psychiatry, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284
- RAYMOND C.A. CHEN, Department of Pharmacology, Peoria School of Medicine, University of Illinois at The Medical Center, Peoria, 1400 West Main Street, Peoria, Illinois 61606
- DORIS H. CLOUET, New York State Drug Abuse Control Commission Research Laboratories, Brooklyn, New York 11217
- GERALD COHEN, Department of Neurology, Mount Sinai School of Medicine, New York, New York 10029
- ALLAN C. COLLINS, School of Pharmacy and Institute for Behavioral Genetics, University of Colorado, Boulder, Colorado 80309
- MICHAEL A. COLLINS, Department of Biochemistry and Biophysics, Stritch School of Medicine, Loyola University of Chicago, 2160 South First Avenue, Maywood, Illinois 60153
- RICHARD A. DEITRICH, Department of Pharmacology, School of Medicine, University of Colorado, Denver, Colorado 80220
- DONALD S. FABER, New York State Research Institute on Alcoholism, 1021 Main Street, Buffalo, New York 14203
- HOWARD J. FRIEDMAN, Center of Alcohol Studies, Rutgers University, New Brunswick, New Jersey 08903
- STEVEN R. GOLDBERG, Laboratory of Psychobiology, Department of Psychiatry, Harvard Medical School, Boston, and New England Regional Primate Center, One Pine Hill Drive, Southborough, Massachusetts 01772
- AVRAM GOLDSTEIN, Department of Pharmacology, Stanford University, and Addiction Research Foundation, Palo Alto, California 94304
- MURRAY G. HAMILTON, Division of Drug and Alcohol Abuse, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284, and Department of Pharmacology, The University of Western Ontario, London, Ontario, Canada
- MAURICE HIRST, Department of Pharmacology, The University of Western Ontario, London, Ontario, Canada

- ANDREW K S HO, Department of Pharmacology Peoria School of Medicine, University of Illinois at the Medical Center, 1400 West Main Street, Peoria, Illinois 61606
- MANFRED R KLEE Max Planck Institute for Brain Research 6 Frankfurt/ am Main, Germany  
Deutschardenstr 46, West Germany
- WERNER A KLEE Laboratory of General and Comparative Biochemistry National Institute on Mental Health, Public Health Service, Bethesda, Maryland 20014
- PING YEE LAW Departments of Psychiatry and Pharmacology University of California San Francisco, California 94122
- A EUGENE LeBLANC Addiction Research Foundation 33 Russell Street Toronto Ontario Canada M5S 2S1
- DAVID LESTER, Center of Alcohol Studies, Rutgers University New Brunswick New Jersey 08903
- HORACE H LOH, Department of Pharmacology University of California, San Francisco, California 94122
- SHERWOOD C LYNN JR Departments of Pharmacology and Psychiatry University of Texas Health Science Center at San Antonio 7703 Floyd Curl Drive San Antonio Texas 78284
- ALICE M MARSHALL Department of Pharmacology The University of Western Ontario London, Ontario Canada
- J MICHAEL MORRISON, National Institute on Drug Abuse 11400 Rockville Pike Rockville Maryland 20852
- HERBERT MOSKOWITZ Southern California Research Institute, 2033 Pontius Avenue Los Angeles, California 90025
- STEVEN H POLLOCK Division of Drug and Alcohol Abuse University of Texas Health Science Center at San Antonio 7703 Floyd Curl Drive, San Antonio Texas 78284
- CARRIE L RANDALL Department of Psychiatry and Behavioral Sciences, Medical University of South Carolina, Charleston, South Carolina 29401
- DAVID H ROSS, Department of Pharmacology and Psychiatry, University of Texas Health Science Center at San Antonio 7703 Floyd Curl Drive, San Antonio Texas 78284
- N SALEM, JR , Center Brain Research and Biochemistry University of Rochester Medical Center, Rochester, New York 14642
- SATANAND SHARMA Southern California Research Institute 2033 Pontius Avenue Los Angeles, California 90025
- ERIC J SIMON Experimental Medicine, New York University School of Medicine New York New York 10016
- ALFRED A SMITH Department of Psychiatry New York Medical College New York, New York 10019
- BORIS TABAKOFF Department of Physiology, College of Medicine, University of Illinois 901 South Wolcott Street, Chicago, Illinois 60680
- F TAKEDA, Center Brain Research and Biochemistry, University of Rochester Medical Center, Rochester, New York 14642
- ANDREW H TANG, The Upjohn Company, Kalamazoo, Michigan 49001
- JACK E WALLACE, Department of Pathology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284
- KENNETH ZIEDMAN, Southern California Research Institute 2033 Pontius Avenue Los Angeles, California 90025

# **Foreword**

## **Part I—Alcohol\***

Alcohol abuse is one of the greatest problems facing our modern-day society. An estimated 9 million people in the United States alone are alcoholics or problem drinkers. Each year alcohol abuse costs the United States economy an estimated 25 billion dollars and the human suffering exacted by alcohol cannot be translated into monetary terms. As the use of alcohol increases, as more young people take up drinking, the incidence of alcohol-related problems also increases. Clearly there is an urgent need to intensify current efforts of treating and preventing alcohol abuse. To maximize the effectiveness of these efforts, a base of reliable, objective, and scientific facts needs to be established.

The subject of alcohol abuse is exceedingly complex. Alcoholism affects all segments of our society and its amelioration poses a real challenge to people concerned with improving the quality of life. Significant strides have been made to help alcoholic people, but the area of alcoholism is fraught with too much heat and too little light. Too much reliance has sometimes been placed on conventional wisdom and dogma. I point this out not to belittle in any way the significant advances that have been made in the past. Rather, I believe that realistic appraisal of the current state of our knowledge lends a hopeful note to the opportunities that lie ahead.

The way to address problems of alcoholism is to ask the difficult questions and probe deeply for answers. We do not know so many essential facts about alcoholism. What is the nature of addiction? What are the precise effects of alcohol on the brain, the liver, the pancreas, and the heart? We need a much greater emphasis on the effects of alcohol on the central

\* This foreword was prepared by Ernest P. Noble, Ph.D., M.D. in his private capacity. No official support or endorsement by the ADAMHA or NIAAA is intended or should be inferred.



nervous system. We do not yet know the neurochemical bases of the behavioral changes produced by alcohol. What are the mechanisms underlying tolerance, adaptation, and dependence? What is the withdrawal reaction and are there drugs that can mitigate it?

The social and psychological aspects of drinking and alcohol abuse urgently need exploration. What stresses of modern-day life impel certain people toward excessive drinking? What role does drinking play for the poor, for minorities such as Indians, Hispanics, and Blacks, for youth and the elderly? Why are more and more women using alcohol? What will be the results of increased drinking in women for their likelihood of becoming alcoholics and what is the effect of increased drinking among women on their offspring? One of the facts that adds to the complexity of the problem is that alcohol use has been a part of our society for so long. Use of the beverage alcohol is part of our sociocultural heritage and this makes for difficult research.

The National Institute on Alcohol Abuse and Alcoholism (NIAAA) is addressing some of these questions. When I was appointed Director of the NIAAA, I brought with me the clear realization of the high priority that must be given to research. To facilitate the basic and applied research activities of NIAAA, we have had to undergo some reorganization. We have elevated Intramural Research to division status with three proposed branches: Biochemistry, Psychobiology, and Clinical Studies. We have also created a separate Division of Extramural Research with two branches: Epidemiology and Special Studies, and Extramural Studies. This reorganization is fortuitous for a healthy exchange of information and will facilitate the development of a knowledge base for maximally effective treatment and prevention.

Through research it is possible to answer some of the crucial and fundamental questions about alcohol. Many scientists, such as the contributors to this book, have already begun to address the challenging area of alcohol abuse. This volume illustrates the kinds of issues that basic research is concerned with. From reading these chapters, one can clearly see that significant advances are being made in understanding the addictive processes, the nature of dependence and withdrawal, and the mechanisms of tolerance. The similarities and differences between alcohol and other drugs such as the opiates need to be determined. Their delineation will have beneficial spinoffs both for the areas of alcohol abuse and drug dependence.

This volume also illustrates the concern of scientists with establishing viable models for assessing the effects of alcohol. Some of the most basic and fundamental questions about drug actions depend upon refined model systems for answers. The nature of tolerance, for example, needs to be explored at the level of the single cell as well as in the intact animal. With sophisticated model systems, scientists can examine one question at a time

with a high degree of control not possible in studies of man. The knowledge derived from basic studies will lead to progress in the applied sciences.

Readers of this book will discover some of the exciting advances that are being made in basic research on alcohol and drug dependence. In the coming years, we will see continuing advances in these areas. This is a challenging area for investigation, and we must proceed in the spirit of excellence. Endeavors to improve the quality of life for the millions of victims of alcoholism is a task worthy of our most compassionate attention.

ERNEST P. NOBLE

National Institute on Alcohol Abuse  
and Drug Addiction  
Rockville, Maryland 20852

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# Foreword

## Part II—Opiates

The last few years have produced exciting and important new developments in opiate research as the result of various outstanding studies by investigators in a number of different laboratories. The seemingly puzzling demonstration that electrical stimulation-produced analgesia in rats could be counteracted by injection of the narcotic antagonist naloxone, in the absence of any exogenous narcotic, was one of the first suggestions of an endogenous opioid action. *In vitro* binding studies with radioactively labeled potent narcotics and narcotic antagonists were found to provide an effective technique for determining the tissue distribution and concentration of the opiate receptors. Probably the most active and fascinating new avenue of research has been in the multidisciplinary area of the isolation, synthesis, and pharmacological characterization of the various endogenous peptides, such as the enkephalins and other endorphins, which apparently act as ligands for opiate receptors. Although at the present time it appears that the endorphins probably produce tolerance and dependence similar to morphine itself, and from a practical standpoint would lead only to a more expensive morphine-like substance, the vast potential of molecular modification may still allow for the synthesis of new derivatives with less addictiveness, greater resistance to hydrolytic or other types of inactivation, longer duration of action, and improved effectiveness by intravenous and other routes of administration outside the central nervous system. Recent work suggests that some of the beneficial analgesic effect of acupuncture may be related to the action of the endogenous opioids, as indicated by the fact that naloxone has been reported to decrease acupuncture analgesia. The expanding studies in the field of the endogenous opioids may lead to the elucidation of many mechanisms of action which are of fundamental pharmacological importance.

Other recent studies have resulted in advances in the understanding of opiate neurochemistry, the nature and purification of opiate receptors, possible differences in opiate receptors, the complex effects of narcotics on behavior, and metabolic aspects of opiate agonists and antagonists. The

following articles present new contributions in many of these areas of opiate research and provide additional scientific knowledge which should assist in the efforts to achieve solutions to the problems of opiate abuse and addiction, and at the same time improve the safety of the valuable legitimate medical uses of the opiates and opioid narcotics.

HAROLD BLUMBERG  
Department of Pharmacology  
New York Medical College  
Valhalla, New York 10595

# Preface

The complexities of contemporary life shape alternatives for man that are frequently bewildering and often frustrating. As a social pharmacologist, I am aware of the deep and divisive social problems that arise from the abuse of alcohol and opiates, both on the macrosocial level and on the level of the family unit and the individual personality. I am also acutely conscious of the human tragedies, the shattered family relationships, the chaotic breakdown in interpersonal communications and understanding, and all the rest of the drug and alcohol abuse phenomenology that tears asunder the fabric of normal social relationships.

It is perhaps an oversimplification to state that before we can achieve adequate means of coping with society's problems of alcohol abuse and drug dependency, we must focus our efforts on the need for scientific investigation and the analysis of scientific data relating to all aspects of the neurochemical and behavioral mechanisms involved. It is a valid observation that there has been far too much heat and too little light generated in this area since the emotional revelations and excitement of the "psychedelic" 1960s and early 1970s.

Basic research can provide the key to understanding the similarities and differences that exist between alcohol and other drugs. While there may be no ultimate answers contained in the findings presented in the following chapters, it is clear that the sheer juxtaposition of alcohol and opiates, viewed both from the neurochemical and behavioral perspectives, represents a "state-of-the-art" look at a comprehensive cross section of recent scientific inquiry. It is an interdisciplinary approach with value not only in terms of scientific content, but also as historical record.

The idea for this book was an outgrowth of a symposium I had the good fortune to chair at the National Drug Abuse Conference held in New York during May, 1976. Thanks to the encouragement and far-sighted planning of Drs. Joyce Lowenson and Frank Seixas, the symposium on "Neurochemical and Behavioral Mechanisms of Alcohol and Opiates" drew multifaceted expertise across a wide spectrum of scientific disciplines, and focused attention on a growing body of knowledge that deserves wider circulation.

This book represents a personal "breakthrough" of sorts, in that it is

the first attempt we know of to compile a comprehensive overview that addresses the commonalities and distinctions of alcohol and opiates, within a framework both of neurochemical and behavioral mechanisms. While the book is not written for the unsophisticated in scientific investigation and inquiry, I am hopeful that this endeavor and following works may eventually provide the kind of scientific base needed on which to build clinical programs designed to alleviate the sociological and medical ills generated by alcohol and opiate abuse and drug dependency. Perhaps such efforts may also lead someday to correcting some of the misunderstanding and myth-based ignorance that prevails in the formation of society's opinions and prejudices regarding the drug and alcohol abuser. After all, unless scientific inquiry, which serves the truth, leads ultimately to public enlightenment, then to what end do we labor?

KENNETH BLUM

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To Arthur H. Briggs, chairman of the Department of Pharmacology at the University of Texas Health Science Center, I express my gratitude for his active support and encouragement of this publication effort.

Dr. David H. Ross must be singled out for his meticulous critique of certain manuscripts submitted, and so must my proofreaders who include Eleanor K. Meyer, Sanford L. Futterman, and Carol Green. My thanks also for the journalistic assistance of my friend Jim Tilton.

Since I am a career teacher in drug addiction sponsored by a grant from the National Institute on Drug Addiction, I also wish to acknowledge not only the sponsorship but the active encouragement of James Callaghan, the monitor of my award with NIDA 1-T01-DA00290-03.

The late Drs. Milton Gross and Michael Walsh must be recognized for their motivational impact on this area of investigation. While Dr. Gross was my mentor in the treatment area, Dr. Walsh's work with Virginia Davis laid the foundation for the initial scrutiny of alcohol—opiate links in a paper they published in 1970.

Speaking of mentors, I cannot forget the men who gave so much of themselves to me in my formative years—Drs. Joseph Seifter and Irving Geller, who showed the way and set the example.

Although there are many others who should be thanked for their part in making this book possible, let me complete these acknowledgments by expressing my heartfelt gratitude to Diana Bard and Murray Hamilton, as associate editors who answered my many demands.



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# **Alcohol and Opiates**

NEUROCHEMICAL AND BEHAVIORAL  
MECHANISMS

# 1. A CRITICAL REVIEW OF PROGRESS TOWARDS AN ANIMAL MODEL OF ALCOHOLISM\*

Howard J. Friedman and David Lester

Center of Alcohol Studies, Rutgers University, New Brunswick,  
New Jersey, 08903.

## INTRODUCTION

Various sets of criteria for an animal model of alcoholism (1,2,3) have been published. Central to each is the requirement that substantial oral ingestion of alcohol be produced. Two of the sets of criteria (1,3) specify that drinking ought to produce measurable intoxication and not be associated with food deprivation; in addition, they stipulate that the animals should overcome obstacles (ranging from merely performing work to facing aversive consequences) to obtain alcohol, sometimes referred to as psychological dependence (1). The only other criteria, besides oral ingestion, which is common to all three are the consequences of chronic alcohol consumption: physical dependence and withdrawal. Other requirements which are not common among the sets of criteria specify that animals consume alcohol to prevent withdrawal (1) or that subsequent to withdrawal the animals re-initiate consumption so that intoxication and dependence are again produced (3).

Some experimental work has been conducted concerning the effect of physical dependence on ethanol selection. Deutsch and Koopmans (4) infused approximately 9 g/kg/day of ethanol into the stomachs of rats continuously for six days; alcohol selection was enhanced thereafter for fourteen days. Physical dependence was not reported, but the intake, a mean of 4 g/kg, would not have been enough to sustain dependence. In any case, these results have not been replicable (Lester et al. unpublished data, 1974, 1975). Myers et al. (5) were unable to produce any increase in voluntary alcohol intake in monkeys which had been made dependent by intubation. Because the testing took place two days after intubation had ended, the procedure could not evaluate drinking to avoid withdrawal

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but only the effect of a period of dependence on later consumption. Begleiter (6) presented an alcohol choice to rats after a period of intubation but did not find any significant intake. In this study, it is not clear when the alcohol choice was presented, but since the animals exhibited withdrawal symptoms, we must assume that choices were presented well after the end of the intubation period and too late, therefore, to prevent withdrawal. Hunter et al. (7) found a significant increase in alcohol consumption in rats given choices between alcohol or sucrose liquid diets during withdrawal. The presentation of the choice before the onset of withdrawal plus the long experience with alcohol consumption undoubtedly contributed to these positive results. Unfortunately, the increase lasted only one to two days and some withdrawal symptoms were observed on the second day. Perhaps a more prolonged increase in consumption could be produced if the procedure also actively built up an association for the animals that alcohol consumption would prevent withdrawal illness; rats exhibited an increased consumption for ten days when they associated ethanol consumption with prevention of thiamine deficiency (8).

It is questionable whether maintenance of drinking to prevent a withdrawal syndrome needs to be a general requirement for an animal model. In some instances alcoholics have undergone partial withdrawal during voluntary administration periods (9,10); likewise, voluntary abstinence and withdrawal symptoms have been observed in rats (11) and monkeys (12). On the other hand, sometimes alcoholics have tried to avoid withdrawal symptoms by tapering off their consumption of alcohol (9,10). Until more is known about the processes underlying physical dependence, the question of the role of physical dependence in maintenance of alcohol consumption will probably remain unanswered.

Much of the research conducted before 1970, which was related to an animal model of alcoholism, seems to have concentrated on inducing consumption of alcohol (13). Little progress has been made in producing pharmacologically significant selective consumption of ethanol (cf., 8). Indeed, some procedures for the induction of alcohol consumption which had previously been regarded as promising, i.e., hypothalamic stimulation (14), intraventricular (15) and intragastric infusions (4), can no longer be so regarded (16,17) (Lester et al., unpublished data, 1974, 1975). Perhaps because of this lack of success the number of articles on this aspect of an animal model has diminished in recent years. In contrast, an increasing amount of research conducted towards the end of contributing to an animal model has concentrated on the aspect of physical dependence and may be due to the development of useful techniques for producing the

required condition. It would appear now to be an appropriate time to assess the work of the last few years and thus this review seeks to evaluate the various techniques used to produce dependence and those used to measure it.

#### *CRITERIA FOR METHODS OF INDUCTION OF PHYSICAL DEPENDENCE*

One should judge any method by its ability to produce objective signs of withdrawal. As Freund (18, p. 314) proposed, an ideal method should do so:

- (a) reproducibly, (b) rapidly, (c) by simple procedures, (d) introducing the fewest variables, (e) by resulting in spontaneous major withdrawal signs and (f) in all treated animals.

An additional, and important criterion, should be the accurate control of alcohol doses, particularly in such a manner that a scale of doses is utilized. This requirement will be assessed in the section on quantifying withdrawal symptoms. Thus far no method fulfills all the criteria mentioned.

#### *METHODS FOR PRODUCTION OF PHYSICAL DEPENDENCE*

A review by Mello (19) provides an excellent survey of this area. This section reviews the additions to the literature since then.

##### Intubation methods

Slight changes in intubation methods from one study to another result in numerous, somewhat different procedures.

Cannon et al. (20) started the intubation schedule in rats with a large (6 g/kg) priming dose. Every eight hours thereafter the animals received supplemental doses of 0-3 g/kg, depending on whether the animals were ambulatory, conscious or neither. Administration continued for two days in one group and three days in another. All animals showed symptoms of withdrawal (tremors and excitability) approximately fourteen hours after the last dose and seven of the twelve animals also showed some form of seizure activity in response to auditory stimulation (key shaking and banging a tin plate with a spoon). No food or fluid was available throughout the administration period. Consequently, the experimental groups and the isocalorically treated controls lost about 15% of body weight during this period. The three day ethanol group lost significantly more weight than the two day ethanol group and their own pair-fed control group.

Intubations every eight hours were also used by Mucha et al. (21), but the initial doses were 1 g/kg. Doses were

increased by 0.2 g/kg if the previous dose did not produce intoxication one hour after intubation, otherwise the previous dose was continued. To compensate for weight losses, if a rat's weight dropped below 90% of its initial weight, it received Metrecal supplements. This regimen was given to different groups for seven, fifteen or thirty days. Withdrawal symptoms, such as hyperreactivity, convulsive activity and audiogenic seizures were found to various degrees among the groups; the interpretation of these differences will be covered in a following section. Seven days of treatment by this procedure did not produce a full range of withdrawal symptoms, for a significant amount of seizure activity was not found. Although care was taken, by means of Metrecal supplements, that body weight was maintained within 90% of initial weight, no compensation was made for the normal growth and weight gain evidenced by the control group. The alcohol animals were, on the whole, 11% below the control animals, and those animals on the regimen the longest, the thirty day group, were 14% below.

Majchrowicz (22) used high doses of ethanol so that dependence was produced in a shorter time. The rats were all begun with a priming dose of 5 g/kg. Over the next four days they received daily doses of 9-15 g/kg divided into 3-5 fractional doses. The actual amount an animals received depended on its degree of intoxication. Unfortunately, due to the adjustment of doses and fluctuation of the number of doses (the exact intervals between doses and the reason for changes being unreported) every animal was treated somewhat differently, and replication would appear difficult. Hunt (23) also used a variable dose method; the doses ranged from 11-15 g/kg/day for seven days. One group received two fractional doses and another three doses per day. The 3-dose/day group tended to have greater changes in seizure threshold during withdrawal than the 2-dose/day group, but the statistical significance was not tested. In both the Majchrowicz (22) and Hunt (23) studies no mention is made of weight losses during ethanol treatment or of procedures to control for this. However, since substantial weight losses were found with methods which used lower doses of alcohol (20) or tried to limit weight loss (21), it must be assumed that these studies were additionally complicated by severe weight losses.

Hillbom (24) and Noble et al. (25) used intubation methods which avoided the complication of variable doses and intervals. Noble et al. (25) started every rat with a total daily dose of 5 g/kg and increased the dose every other day by 1 g/kg until a dose of 10 g/kg was reached; the daily dose was given in two equal portions every twelve hours. Hillbom (24) began with doses of 4-5 g/kg per day and

continued at that level for ten days. Thereafter, the doses were increased every other day by 1 g/kg to a final dose of 10 g/kg; the doses were divided into two portions every twelve hours. In both studies a non-alcoholic liquid diet was available as the sole source of food and fluid. These procedures prevented weight loss during treatment (24,25) and enabled almost normal weight gain (24). The lack of weight loss seemed, in large part, due to the use of the liquid diets. If animals were maintained on solid lab chow, they lost a significant amount of weight (25). The animals in both studies displayed withdrawal seizures after ethanol administration ceased.

### Polydipsia

This procedure has been proposed by its developers (2) to be an ideal animal model of human alcoholism and to satisfy the criteria they themselves proposed, criteria not as complete as those outlined by Cicero and Smithloff (1) and Lester and Freed (3). In any event, Deutsch (26) argues that polydipsia does not in fact fulfill the criteria advanced by Falk et al. (2). As with every other method of producing dependence, the animals were not voluntarily ingesting alcohol. Since polydipsia is produced by a process which is not understood (27), it seems hardly possible for the authors to state flatly that the drinking is not dependent on any contingencies. In polydipsia, excessive alcohol intake appears moderated by the calories provided by alcohol, the pharmacological properties being of secondary importance (28). Finally, this procedure did not produce dependence when an attempt to replicate the paradigm was made (29).

### Inhalation

The method developed by Goldstein and Pal (30) has been criticized (19) for its use of pyrazole and for weight loss during inhalation. A modification used by Griffiths et al. (31) overcomes these problems. Ethanol was administered to mice for a longer period, ten days, and the ethanol vapor concentration was gradually increased from 10-15 mg/L on day one to 25-35 mg/L on day ten (31) rather than three days with alcohol exposure of 10-16 mg/L (30). This method of gradual increase in vapor concentration resulted in less mortality, 10% vs. 50%, than when no pyrazole was used (32) and in no significant weight loss (31). No data were presented to indicate whether or not the fluctuations in blood alcohol concentrations, which pyrazole was used to prevent, were under control. Presumably, since mortality was decreased, the fluctuations were attenuated. Due to the more rapid rate of

ethanol elimination without pyrazole, withdrawal symptoms occurred sooner. One puzzling aspect of this study is that the control animals had appreciable seizure scores themselves: possibly these mice, T.O. strain, are prone to seizures, with alcohol accentuating this factor.

Roach et al. (33) maintained rats for seven days in an ethanol atmosphere of 15-30 mg/L. No pyrazole was used. Severe withdrawal symptoms were produced, including convulsions when suspended by the tail, and the relative intensity of the withdrawal symptoms was related to the mean daily blood alcohol level, as with mice (32). A drawback of the procedure is that the mean daily blood alcohol levels (and daily alcohol intake) were outside of the control of the experimenter, but were dependent upon each animal's ventilation and metabolic rates. Presumably, individual blood alcohol levels fluctuated considerably; no mention was made of mortality, and weight losses, averaging 20%, were not prevented with this procedure.

French and Morris (34) maintained rats in chambers containing low concentrations of ethanol vapor, 1.4 mg/L for two weeks. Not surprisingly, there were no detectable blood alcohol levels in the rats. Whether this procedure truly produced dependence is questionable. None of the usual behavioral symptoms of withdrawal were evident. The only symptoms were a decrease in weight one day after withdrawal and a slight increase in reactivity only after seventy-two hours; the body weight decrease was not tested for significance.

#### WITHDRAWAL MEASURES

Withdrawal symptomatology has been described for a wide range of animal species: monkey (12,35), chimpanzee (36), dog (37,38), rat (22), and mouse (30,39). The withdrawal syndrome of the higher animals, i.e., monkeys, chimpanzees and dogs, more closely resembles the human syndrome, with clinical symptoms such as alkalosis and hallucinations observed in these higher species (12,35,37). However, these observations have been at best semi-quantified with the variety of symptoms grouped into three categories judged to be of increasing severity (40) and at worst only qualitative, with the various symptoms present merely listed (12,36,37,38). One should also note that withdrawal symptoms in humans, such as changes in body temperature, heart rate and blood pressure, should be quantified so that better comparisons to the animal data are available (41).

Scales for ranking withdrawal symptom severity have been developed with rodents. Some of the scales are only semi-quantitative: the symptoms are divided into broad stages of differing severity and the particular stage in



which an animal is placed depends on a non-instrumental observation (11,22,39,42,43). These methods have several disadvantages. Perhaps the most obvious is simply the amount of time which must be spent in observing the animals for withdrawal symptoms with no assurance that, despite the large investment of time, every occurrence will be observed. Also, no allowance is made for accurately comparing the severity of the various symptoms between animals: an animal which has one convulsion is rated equal to an animal which has ten. Since these methods regard withdrawal as a continuum, as a series of symptoms of increasing severity, each following the preceding ones, a symptom which occurs early, such as hyperactivity, but is very intense, would be overshadowed in the rating by a later symptom, such as whole-body rigidity, which might be very mild. It would probably be best to rate the severity of each symptom rather than merely note its presence or absence. Replication becomes a problem with those methods which arbitrarily label an occurrence of a symptom as mild, intermediate or severe (22,42). Another researcher would find it formidable to rate intensities similarly. Mucha et al. (21) attempted to assess severity by observing the animals over several short observation periods, counting the number of periods in which each of several withdrawal symptoms appeared. However, this count is still neither a reliable measure of severity nor even of frequency: whereas an animal only scores zero or one for a ten minute observation period, several intense bursts of forelimb clonics will score equally with one instance of head-shake. On the other hand, a positive aspect of this method is that the three symptom categories are counted separately. Although the withdrawal symptoms are displayed in a definite order, it is probable that the various ethanol withdrawal symptoms, just as morphine withdrawal symptoms (44,45), are mediated by different neural systems. Experiments testing modification of withdrawal would, thus, be better served by a rating method which treats the symptoms as separately modifiable. In addition, the impossible task of determining whether intense hyperreactivity is more severe a symptom than intermediate whole-body rigidity (or vice-versa) is avoided.

### Separate Symptoms

#### *Convulsions*

The most commonly studied symptom has been convulsions, evoked or spontaneous. Generally, convulsions are scored simply on the basis of how many animals have seizures in response to a sound, usually a specified bell (21,24,46,47,48). Some investigators (2,11,20), however, persist in using keys,

an "instrument" which can hardly be conceived of as scientific or standardized: the "instrument" is certainly not reproducible. Simply recording the number of animals having seizures does not measure differences in severity.

Since there are several aspects of seizure, the different gradations can be scored. Goldstein (32) devised a five level scale for grading seizures elicited by suspending mice by the tail. After multiple tests over time, a curve is plotted and both the peak height and the area under the curve serve as measures of severity. This method has been found to be reliable and replicable by others (31,49). Similarly, seizure responses have been graded in rats; Ratcliffe (47) and Noble et al. (25) both used six level scales, but the categories were not identical. Since the two studies used different eliciting stimuli, a bell by Noble et al. (25) and electroshock by Ratcliffe (47), different seizure patterns may have resulted, thus necessitating the particular categories. Various other scales (50,51) which have been developed to rate audiogenic seizures can also be applied to ethanol withdrawal testing. Ratcliffe (47) also measured the duration of the seizures in response to bell ringing; the reliability of this measure has not been determined because the small number of animals did not permit statistical tests.

Hunter et al. (52) and Walker and Zornetzer (53) compared changes in EEG activity with behavioral changes during withdrawal in rats and mice respectively. EEG events were found not to be correlated with behavioral symptoms of withdrawal. Convulsions could be elicited while EEG events were at pre-convulsive stages indicating that cortical activity is not a reliable indicator of behavioral hyperexcitability.

An alternative to measuring variations in response is, of course, measuring changes in threshold, the changes in level of stimulus needed to produce a criterion response. McQuarrie and Fingl (54) measured changes in threshold for electroshock seizures in mice. A decrease in threshold, a hyperexcitability, reached its peak fully two days after the last dose of ethanol; the threshold returned to normal five days later. A disadvantage of this method is that large numbers of subjects must be used. Since an individual animal's threshold is affected by a sub-threshold stimulus (55), additional animals must be used for successive stimulus levels. Drug stimuli, such as leptazol, strychnine (47) and pentylenetetrazole (23), enable the same animal to be injected with successive increments of convulsant agent until the criterion response is obtained. Thus, a threshold is determined directly for each animal, rather than a calculated value at which 50% of the animals would have responded. Hunt (23) found, as did McQuarrie and Fingl (54), that seizure threshold decreased on the first day of withdrawal. However,

on succeeding days threshold for pentylenetetrazole-induced seizures increased above normal levels (23) whereas McQuarrie and Fingl (54) had found a further drop followed by a gradual return to normal.

### *Reactivity*

Hyperreactivity can also be measured in either of two ways: a change in stimulus threshold needed to produce a criterion response or a change in response magnitude after a constant level stimulus. Gibbons et al. (55) found a decrease in threshold of foot shock needed to produce both a flinch and a jump response in rats after a period of alcohol deprivation. The hyperreactivity measure seems to be a rather sensitive measure of withdrawal since the alcohol administration procedure used, once daily intubations of ethanol which increased from 3 to 7 g/kg (56), produced a relative paucity of other withdrawal symptoms in comparison to more frequent schedules (23). A semi-quantitative variation of a threshold method was used by Hillbom (24). A wire heated to one of three different temperatures was placed on a rat's tail; tail movement within sixty seconds was recorded as a positive response. Inexplicably this test found behavioral hypo-reactivity, which is at odds with other reports of withdrawal symptoms. In any event, the test, as used, gives no indication of any threshold changes since the results of all the heat levels were reported together; there was no grading of the response. Thus, the test merely reported the presence or absence of a response to what was essentially a unitary stimulus.

A test of hyperreactivity used by Mucha et al. (21) measured changes in response, but did so only semi-quantitatively. Rats were judged as being hyperreactive if they resisted handling more than normally; a simple positive or negative response was recorded. The animals were tested thusly every other hour for sixteen hours. Because of the lack of a response magnitude this test seems to be more a measure of duration of withdrawal hyperreactivity than of withdrawal severity. French and Morris (34) utilized a simple apparatus to quantify response magnitude. Rats were given foot shocks of six different intensities, ranging from 0 to 0.5 mA, while suspended in a scale; the deflection in grams of the scale was taken as proportional to the response magnitude. However, with the alcohol dosage used, this method detected no changes in reactivity during the first day of withdrawal, and it was not until seventy-two hours afterwards that any significant hyperreactivity was detected. Given the trivial amounts of alcohol these rats received (not more than 1 g/kg/day), it may be doubted that these

results are meaningful or replicable. Pohorecky et al. (57) obtained changes in reactivity in rats which had a time-course similar to that which has been grossly observed during withdrawal (11). The magnitude of rats' startle responses to a buzzer was measured by means of a stabilimeter which produced a change in electromagnetic field proportional to an animal's movement; the characteristics of the buzzer were, unfortunately, not reported.

### *Body Weight*

Several investigators have found body weight loss to be a reliable symptom of ethanol withdrawal in various species. Essig and Lam (38) found weight loss in dogs throughout the first eight days of withdrawal. Cannon et al. (20) found a significant absolute weight loss in treated rats thirty-two hours following the last alcohol dose. It might be expected that the more severe the dependence and, hence, the withdrawal, the larger the withdrawal weight loss. However, this study (20) is not in agreement with such an expectation: the animals which had a shorter ethanol treatment period, two versus three days, had a larger withdrawal weight loss. This result may have occurred because the groups were not at the same weight at the start of withdrawal. Those animals treated for three days had lost significantly more weight during the treatment period and, thus, had less available weight to lose during withdrawal. Goldstein and Kakihana (57) found that, when weight loss was presented as proportion of weight loss during withdrawal, weight changes in mice paralleled other indicants of withdrawal severity. DBA mice had both higher seizure scores (32) and had greater weight losses than C57BL mice. Weight loss is quite possibly a very sensitive measure, for even those C57BL mice which had no seizure responses did show weight changes. Indeed, it is surprising that body weight changes have received such little attention in alcohol withdrawal; besides its simplicity, this method has been shown to be an excellent indicant of morphine withdrawal (59,60).

### *Activity*

Spontaneous activity changes during withdrawal have been measured in both rats and mice. Hunter et al. (11) derived hourly composite activity scores from observations of grid crossings, rearings and grooming of rats for the first eight hours of withdrawal. Although there was substantial variability, it appears that there was a period of increased activity before the blood alcohol concentration reached zero; a phase of reduced activity followed. However, it is im-

possible to determine if the effects are significant since there were no statistics and no control or baseline scores. Cicero et al. (61) reported increased activity in most rats which had been maintained on ethanol. However, the results are not comparable to those of Hunter et al. (11) since the measure was open-field activity, and testing commenced twenty-four hours after the beginning of withdrawal. It should be noted that Cicero et al. (61) found that some of the animals displayed decreased rather than increased activity. This finding is implicit in the variability of activity found by Hunter et al. (11), and the results together indicate that neither activity change is an exclusive symptom of withdrawal in rats. Increased activity, as measured by an electromagnetic activity platform, was found in mice during the first six hours of ethanol withdrawal (31). However, once again no statistics were presented so that the significance of the changes is unknown. No study has yet demonstrated that any particular activity change is correlated with withdrawal nor what the time course of such changes might be.

### *Tremors*

Tremors are probably the most widely cited symptom of withdrawal, but only in a qualitative sense: either they are present or absent. Mello (19) raised the possibility of using certain tremor analysers (62,63) to quantify ethanol withdrawal tremors. Freund (64) described a procedure whereby mice were housed in a jar suspended from a muscle transducer so that the duration, frequency and amplitude of both tremors and seizures could be recorded. However, no data have yet been presented to assess this stratagem.

### *Temperature*

It is surprising that body temperature changes, as with weight changes, have been so rarely investigated. Temperature changes, besides being evident during human ethanol withdrawal (65) are a good indicant of morphine withdrawal (45). Tabakoff (66) found hypothermia in mice undergoing withdrawal which was proportional to the duration of the ethanol administration period; temperatures were measured every several hours. The time-course of temperature changes paralleled the development of other symptoms. The only other instance of temperature changes found in animals during withdrawal was in chimpanzees, but data were not reported (36). Pohorecky (67) was unable to detect any temperature changes in rats undergoing withdrawal; this difference from the Tabakoff (66) findings might have resulted from the temperatures being recorded at about four hours after the peak of withdrawal

(twelve hours vs. eight hours) or simply a species difference. In any case, a change in preference of environmental temperature was found. Animals were placed in a T-maze which had the alley at ambient temperature, one arm at 40° C. and the other arm at 4° C.; the amount of time spent in each location during a five minute trial was recorded. Animals withdrawn from alcohol increased their preference for the warm arm; the peak of this behavior was found seventy-two hours after ethanol. The results were interpreted as a change in set-point for temperature, a measure more sensitive than body temperature changes.

### *Aggression*

Enhanced aggression is another measure of withdrawal which appears applicable to ethanol withdrawal. To measure aggression threshold, pairs of rats can be given increasing levels of foot-shock, five shocks at each level, and the levels at which vocalizations, rearings, and biting attacks occur, recorded. A decrease in threshold for shock-induced aggression was thus found seventy-two hours after the beginning of ethanol withdrawal (Lal, personal communication, 1975), but not at forty-eight hours, a time course identical to that of morphine withdrawal (68).

Up to now we have been labelling the methods which determine numerical scores for withdrawal symptoms as quantitative. Let us now consider just how quantitative they are. What is the basis for believing that a lower score means less severe withdrawal? Exactly how much less severe is a low score compared to a high score? In the numerous articles which report measuring withdrawal symptoms the majority merely compare the withdrawal scores of a control group and a single alcohol treated group. These studies can hardly be said to have determined how reliable their particular withdrawal measures were for distinguishing differences in severity of withdrawal. For any withdrawal measure to reliably determine differences in severity, that measure must be applied to groups of animals which are differentially dependent; in other words, a dose-response function must be determined. Using an inhalation method of alcohol administration, Goldstein (32) has indeed determined such a function. Mice were exposed to several combinations of different ethanol vapor levels and different durations of ethanol inhalation. The different ethanol vapor levels produced different blood alcohol concentrations. Measuring withdrawal by scoring handling-induced seizures detected differences in intensity of withdrawal which were linearly related to the time-integrated dose of alcohol. Thus, an animal which had a blood alcohol concentration (BAC) of 1.14 mg/ml for three

days received approximately the same total dose (3.42 mg/ml X days) as an animal which had a BAC of 0.28 mg/ml for thirteen days (3.64 mg/ml X days), and both had the same intensity of withdrawal seizures. Doubling the total dose of alcohol approximately doubled the withdrawal score.

The ability to determine a dose-response function seems to depend on the method of alcohol administration. Those studies which tried to vary the degree of dependence using oral administration procedures, for the most part, were not able to obtain as plausible dose-response functions as found by Goldstein (32). Cannon *et al.* (20) did not find a difference in incidence of seizure between groups of rats intubated every eight hours for either two days or three days. The other measure used, body weight loss, was the reverse of what would be expected; as mentioned before, the two day group lost more weight during withdrawal than the three day group. Mucha *et al.* (21) also intubated rats every eight hours but for longer periods: seven, fifteen and thirty days. The semi-quantitative withdrawal measures of hyper-reactivity and convulsive activity detected differences in withdrawal severity only between the seven and thirty day groups; no differences were found between either seven and fifteen day groups or fifteen and thirty day groups. Another measure, incidence of audiogenic seizures, found no differences between either an untreated group and the seven day group or between the fifteen day and the thirty day groups. Of course, it is possible that the measures used were not sufficiently sensitive or accurate to detect differences in severity. However, aspects of these intubation procedures inherently weaken their ability to serve as models for quantification of withdrawal severity. All the animals within a group did not receive the same amount of alcohol because each dose which an animal received was raised or lowered dependent on its degree of intoxication (20,21). This is not to imply that all intubation procedures are unfeasible. It is simply necessary that all animals within a particular treatment group receive the same treatment, *e.g.*, the intubation procedure used by Noble *et al.* (25).

Another administration procedure, liquid diet, seems generally ill-suited to determinations of a dose-response function. The duration that animals are on the diet is, of course, controlled by the experimenter, but the actual amount the animals consume is not under the experimenter's control. Animals often manage, perversely, to utilize opportunities to obfuscate experiments: thus Hunter *et al.* (11) maintained rats on a liquid diet for ten, fifteen, twenty or thirty days. Spontaneous abstinence periods were observed. As the duration of the alcohol period increased both the number of animals spontaneously abstaining

and the number of times individual animals abstained increased. Thus, a reliable scale of doses became unobtainable.

Determining a dose-response function is, therefore, a *sine qua non* for determining how accurate withdrawal measures are and for determining how reliable the alcohol administration procedure is. As mentioned above, such a function can be obtained by recording of total alcohol intake; frequent determinations of blood alcohol levels, and the subsequent calculations of a time-integrated function, may also produce an accurate dose-response curve. In either case, it appears important that no discontinuity in the intake of alcohol occur.

#### SUMMARY

That physical dependence and an abstinence syndrome can result in animals after substantial alcohol intake is beyond dispute. Scientific elegance and analysis would be served if the variety of withdrawal symptoms were each separately quantified; such quantification will make it possible not only to better assess the relation between dose and response, but also to assess such treatments as may modify one or another of the dysfunctions of the withdrawal period. Of the various procedures used for giving animals alcohol, we are convinced that only such procedures which give the experimenter real control of the amounts of alcohol obtained by the animals can be valid; even with such a method (e.g., intubation), it may be essential to establish the equality of the dosing level within a group by measuring their time-integrated blood alcohol levels. Observance of these strictures of experimental detail should lead to a comprehensive description of the dose-response function and, reasonably, to a better understanding of the central effects of alcohol.

Although the various facets of an animal model have not yet been put together, the techniques which may be useful in its assemblage appear at hand. The methods described by which voluntary alcohol consumption can be increased may prove to meet the initial criteria advanced for an animal model in specific mouse or rat strains. Mouse or rat strains which either "prefer" an alcohol solution (C57BL) or have been bred for this purpose [the AA rat strain (69)], the long or short sleep mice bred by McClearn (70) (see also 71) or the rat strains bred for disparate impairment of motor activity in response to alcohol (72) might well be prime candidates for an animal model. The quantification of the withdrawal symptomatology should also make it possible to assay with precision whether the experimental modifications producing increased voluntary consumption of alcohol do in fact lead to physical dependence of any significant degree.



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## 2. NEUROCHEMICAL ASPECTS OF ETHANOL DEPENDENCE\*

Boris Tabakoff

University of Illinois Medical Center, Department of  
Physiology, 901 South Wolcott Street, Chicago, Illinois,  
60680.

### INTRODUCTION

The neurochemical components of the addiction syndrome referred to as alcoholism, most probably result from the interaction of this drug with neurons which under normal circumstances are tightly controlled by feedback regulatory systems. The presence of ethanol in the cellular milieu for extended periods and the resultant alterations in neuronal function may produce a resetting of the regulatory systems to compensate for the presence of ethanol. Such a conceptualization of the effects of ethanol on control systems is similar to the conceptual framework within which the study of other addictive agents has been carried out (1,2). The prior theories relating to the development of physical dependence have envisioned a continuum between the development of tolerance and physical dependence. However, as will be discussed in the following paragraphs and as previously noted by Kissin (3), the development of tolerance and physical dependence to ethanol may not be due to a common singular mechanism. Tolerance and physical dependence may, however, be causally related phenomena; i.e., the development of tolerance in a susceptible individual may simply allow for imbibing of sufficiently large doses of ethanol such that physical dependence can become manifest.

### NEURONAL FUNCTION

Dependence and tolerance have been studied on various levels of physiological complexity. At the neuronal level,

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chronic consumption of ethanol has been thought to alter the cybernetic capacity of the cells. If one considers that the final information-containing event in neuronal transmission can be quantitatively expressed as the amount of transmitter released at terminals (4), one can easily envision several points of interaction where ethanol may alter such output. The interactions between ethanol and the neuron may be direct or indirect. Direct interactions are exemplified by the interaction of ethanol with cellular membranes (5), which can lead to a block in neuronal conduction (6). The ability of ethanol to block conduction seems to be inversely related to the size of the neuron (7) and one should consider that this selective action of ethanol also imparts a concentration-dependent specificity to the actions of ethanol. Other direct effects of ethanol may be exemplified by changes in receptor sensitivity induced by ethanol (8). The receptors affected may be the post-synaptic excitatory or inhibitory receptors, or the recently described presynaptic receptors which control the overflow release of transmitter (9). On the other hand, the effects of ethanol may be indirect such as those producing changes in input to a particular neuron (10). Inputs travelling multisynaptic paths, as those determining evokes cortical potentials, seem to be more susceptible to the actions of ethanol (7) than inputs travelling direct paths. All the above examples lead one to expect that the interaction between ethanol and neurons would change the output of neurons, and such changes in output would activate feedback controls which would try to compensate for the effects of ethanol. One can then expect that the examination of changes in the turnover of the various neurotransmitter substances in the CNS during chronic ethanol treatment and during withdrawal, may lend insight into the functional changes responsible for the development of tolerance and physical dependence.

#### *THE "ANIMAL" MODEL FOR ANALYZING THE EFFECTS OF ETHANOL ON BRAIN FUNCTION*

The necessity for examining, chemically, brain material during the development of physical dependence to ethanol has led to the recent proliferation of animal models of the disease (11,12). Certain criticisms have been leveled at the applicability of such models to the study of alcoholism, since some authors have experienced difficulty in establishing drug-seeking behavior in animals when ethanol is used as a positive reinforcement (13). However, this difficulty was not evident in all studies and ethanol has been demonstrated by other investigators to be a strong reinforcer of behavior (14) leading to intake of increasing amounts of

ethanol (15). Although controversy remains regarding the induction of alcohol-seeking behavior in animals, it has been clearly demonstrated that animals do become physically dependent on ethanol if dependence is defined in terms of the occurrence of withdrawal symptoms after removal of ethanol from the animals' diets (11). Many of the overt symptoms evidenced during the early period of withdrawal in animals bear a resemblance to the symptoms seen in humans (Table 1).

TABLE 1  
SYMPTOMATOLOGY ASSOCIATED WITH ACUTE (EARLY)  
WITHDRAWAL SYNDROME

<u>Humans</u>	<u>Animals</u>
tremors	tremors
anorexia	anorexia
insomnia	EEG manifestations
REM rebound	convulsions
convulsions	temperature aberrations
temperature aberrations	agitation
agitation	piloerection
hallucinations	mydriasis

Human withdrawal symptomatology has been separated by some authors (16) into an early phase and a late phase known as delirium tremens, and concordant with such a progression, French and his coworkers (17) have also reported a secondary phase of withdrawal in animals. This phase included hyperkinesia which developed approximately three days after ethanol withdrawal, and was equated by the authors to the delirium tremens state of human withdrawal. In the past, one of the drawbacks in human and animal experiments has been the lack of continuous quantitative measures of the severity and time course of ethanol withdrawal. Recently a good behavioral and physiologic method of analysis of the human syndrome has been proposed by Gross et al. (18), and we have found that body temperature may serve as a good quantitative means of assessing withdrawal severity in some animals (19). However, one must be careful not to depend on only one symptom to monitor withdrawal, since different biochemical events may be responsible for the various components of withdrawal as exemplified by studies of opiate withdrawal (20). For example Cox et al. (20) found that hypothermia and "wet" shakes occurring during morphine withdrawal were controlled by dopaminergic neurons, while other symptoms such as diarrhea, head shakes and sneezing were not at all affected



by manipulation of the dopaminergic systems in brain.

*ALTERATIONS IN NEUROTRANSMITTER LEVELS AND TURNOVER PRODUCED BY ACUTE AND CHRONIC ETHANOL INTAKE*

Several authors (21,22,23) have attempted to analyze, by use of pharmacologic agents, which neuronal systems are of primary importance in determining the severity of ethanol withdrawal symptoms. These studies have produced somewhat contradictory results. The contradictions within these studies are probably due to the nonequivalence of the symptoms examined by the various authors (21,23). Thus, while the studies of Goldstein (21) and Blum (22) indicated that catecholaminergic neurons may play a determinant role in producing convulsions due to handling (11) in mice undergoing withdrawal, the studies of Collier (23) indicate a primary role for serotonergic systems in determining withdrawal symptomatology. However, Collier (23) monitored head twitching as a primary measure of withdrawal. Alterations in GABA metabolism by pharmacologic agents have also been reported to produce changes in the severity of withdrawal convulsions (21) and this transmitter was suggested as a candidate for determining withdrawal symptomatology. The use of pharmacologic agents (eg. reserpine, picrotoxin) which in themselves predispose animals to seizures, may lead to ambiguous results. The systems affected by the drugs may simply prove to be synergistic with other undefined systems which are actually responsible for the symptoms of ethanol withdrawal. Pharmacologic manipulations should be correlated with actual changes in neurotransmitter utilization occurring in the CNS prior to and during withdrawal before any definitive conclusions may be reached as to which systems are determining the manifestations of physical dependence.

Many studies on both the levels and the "turnover" of various postulated transmitter substances have appeared in the literature and a representative sampling of the conclusions is presented in Table 2. These results indicate a lack of agreement on what changes occur in the various measures of serotonergic, dopaminergic and GABA-dependent function in the CNS of animals treated chronically with ethanol. Some of these differences are probably due to such variables as time of testing and the degree of tolerance and physical dependence developed in the animals. In addition, the necessity of assessing whether animals are still intoxicated or are in partial (24) or total withdrawal is paramount. Another major problem is that different methods of testing neurotransmitter "turnover" in the CNS may in themselves lead to different results. We found this to be the case with measures of serotonin turnover (25). If

TABLE 2

EFFECT ON ETHANOL ON NEUROTRANSMITTER AMINES

Transmitter	Acute Administration		Chronic Administration	
	Levels	Turnover	Levels	Turnover
5-HT	↑ ①		↑ ①	
	↔ ②	↔ ②	↔ ②	↔ ↓ ②
	↔ ③	↔ ③	↔ ③	↑ ③
		↔ ④		↔ ④
	↔ ⑤	↓ ⑤	↔ ⑤	↓ ↔ ⑤
DA	↓ ⑥	↑ ↔ ⑥		
	↔ ⑦	↓ ⑦	↔ ⑦	↓ ↑ ⑦
			↔ ⑧	↔ ⑧
			↔ ↓ ⑨	
	↑ ⑩		↑ ⑩ ⑫	
	↔ ⑪		↓ ⑬	

References

- Phoracky et al., 1974
- Tabakoff et al., 1974, 1976, 1978
- Kuriyama et al., 1971
- Frankel et al., 1974
- Hunt and Majchrowicz, 1974
- Carlsson et al., 1973
- Hunt and Majchrowicz, 1974
- Ahtee and Svartstrom-Fraser, 1975
- Patel and Lal, 1973
- Rawat, 1974
- Hakkinen and Kulonen, 1963
- Sutton and Simmonds, 1973
- Sytinsky et al., 1975

Key:

- ↑ increase
- ↓ decrease
- ↔ no change

serotonin turnover was measured by the method of Neff and Tozer (26) using the monoamine oxidase inhibitor pargyline, no differences in serotonin turnover were evident between ethanol withdrawn or control animals. On the other hand, measuring turnover by monitoring the conversion of administered <sup>14</sup>C-tryptophan to serotonin and 5-hydroxyindoleacetic acid demonstrated a significant decrease in the utilization of serotonin in brains of mice undergoing withdrawal (25). Such disparate results may be explained by the known inhibitory effect of monoamine oxidase inhibitors on discharge

rate of serotonergic neurons (27).

In examining the published results on the effects of ethanol on transmitter turnover, one area of agreement does, however, become evident. An acute dose of ethanol decreases the turnover of norepinephrine (NE) in brain while chronic ethanol administration produces increases in NE turnover which are evident throughout the period of withdrawal (see Tables 3 and 4).

An initial increase in turnover of NE seen after an acute dose of ethanol by Hunt and Majchrowicz (28) (Table 3) may be a result of the early excitant, disinhibiting effects of ethanol in the CNS (29) or possibly due to the release of catecholamines in the CNS (30) by an early infiltration of brain tissue with acetaldehyde. Since the availability of  $NAD^+$  is the rate-limiting step in ethanol metabolism (31), the administration of large doses of ethanol could result in an initial rapid generation of acetaldehyde while  $NAD^+$  is available for ethanol oxidation. The initial increase in NE turnover after acute ethanol administration is, however, soon supplanted by a period of decreased noradrenergic activity which is evident for the remainder of the period of intoxication (28,32,33).

In contrast to the effects of an acute dose of ethanol, chronic ethanol administration results in a prolonged and sustained period of increased NE turnover (Table 4). Whether this increased turnover is responsible for certain of the withdrawal symptoms or is a response elicited by the withdrawal symptomatology remains an enigma. Another possibility is that alterations in NE metabolism may be related to the development of tolerance rather than physical dependence.

#### *MECHANISMS BY WHICH ETHANOL MAY ALTER NE TURNOVER--ROLE OF CALCIUM*

Before speculating on these subjects one should consider the mechanisms by which chronic ethanol intake may alter the functional aspects of NE metabolism. The rate-limiting enzyme in the synthesis of NE is tyrosine hydroxylase (34). The activity of this enzyme is coupled to impulse flow in noradrenergic neurons (35) and to concentrations of certain components (eg.  $Ca^{++}$ , catechols) which comprise the intracellular milieu in which the enzyme resides (36). The enzyme is quite sensitive to feedback inhibition by NE (37), and control of enzyme activity has been postulated to be maintained by changes in a small extravesicular pool of NE during impulse flow (37). Thus, depletion of such a pool of NE would decrease the feedback inhibition and increase the activity of tyrosine hydroxylase. The block in conduction in neurons after administration of ethanol could result in an

TABLE 3

EFFECT OF A SINGLE DOSE OF ETHANOL ON BRAIN  
NOREPINEPHRINE TURNOVER

<u>Dose</u>	<u>Blood Ethanol</u>	<u>Method of Measure</u>	<u>Turnover</u>	<u>(Ref.)</u>
4g/Kg i.p.		<sup>3</sup> H-tyrosine injection	decreased* 90 min after ethanol	(32)
4g/Kg p.o.	363 ± 29 mg%	Metabolism of administered <sup>3</sup> H-norepinephrine	decreased 60 min after ethanol	(71)
5g/Kg p.o.	100-300 mg%	αMe-p-tyrosine	increased** 15 min after ethanol	(28)
			decreased 2 hours after ethanol	(28)

\*Increased accumulation of counts in NE but decrease in metabolite radioactivity over controls.

\*\*This initial increase in NE turnover may be a result of early excitant, disinhibiting effects of ethanol in the CNS.

TABLE 4

EFFECTS OF CHRONIC ETHANOL ADMINISTRATION  
ON NOREPINEPHRINE TURNOVER

<u>Time of Assay</u>	<u>Blood Ethanol</u>	<u>Withdrawal Signs</u>	<u>Turnover (Ref.)</u>
Still intoxicated	250-300 mg%	no	increased (32)
8 hours after withdrawal	0	yes	increased
24 hours after withdrawal	—	no	increased
Still intoxicated	175-300 mg%	no	increased (28)
8-12 hours after withdrawal	0	yes	increased
Still intoxicated	?	no	no change* (32)
16-18 hours after last dose	?	yes	increased*

\*compared to water-intubated controls

accumulation of presynaptic NE and produce a decrease in enzyme activity which would be reflected in the decrease in NE turnover measured after acute ethanol administration. A more current proposal on the control of tyrosine hydroxylase involves the effects of  $\text{Ca}^{++}$  and cyclic adenosine monophosphate (c-AMP) on enzyme activity (36,38). The addition of  $\text{Ca}^{++}$  and c-AMP to assay mixtures for tyrosine hydroxylase has been shown to increase the affinity of the enzyme for its cosubstrates (reduces pteridine and tyrosine) and to significantly decrease the affinity of the enzyme for norepinephrine. The decreased affinity for the natural feedback inhibitor of the enzyme results in an effective increase in enzyme activity even in the presence of NE. Since increases in intracellular  $\text{Ca}^{++}$  and c-AMP can be coupled to impulse flow (39), the levels of these compounds may synchronize enzyme activity with the need for transmitter during neuronal activity. Seeman et al. (40) have shown that a significant amount of  $\text{Ca}^{++}$  is segregated from the free form onto cellular membranes in the presence of ethanol, and this phenomenon could remove the  $\text{Ca}^{++}$  necessary for optimum activity of tyrosine hydroxylase. In addition, Ross et al. (41) have demonstrated that total brain  $\text{Ca}^{++}$  levels are actually decreased after a single dose of ethanol. The decreases in free  $\text{Ca}^{++}$  and total brain  $\text{Ca}^{++}$  coupled with decreased impulse flow could sum to produce the significant decrease in turnover of NE witnessed after a single intoxicating dose of ethanol.

Brain  $\text{Ca}^{++}$  levels have been postulated to be controlled by active transport systems residing in the choroid plexus (42) and our studies (43,44) have shown that ethanol both *in vivo* and *in vitro* inhibits certain active transport systems of the choroid plexus. Although the transport systems shown to be inhibited by ethanol were those removing organic acids from brain, the transport of organic acids has been shown to be coupled to inorganic ion [including  $\text{Ca}^{++}$  (45)] transport within the choroid plexus (46). Thus, the effects of acute ethanol administration on brain  $\text{Ca}^{++}$  levels (41) may be directly related to the inhibition of choroid plexus transport systems by ethanol (43).

To account for the increased NE turnover after chronic ethanol intake, one would have to predict that a tolerance develops to the membrane and  $\text{Ca}^{++}$ -lowering effects of ethanol. Interestingly, Ross and coworkers have recently reported (47) that chronic exposure of animals to ethanol results in increased  $\text{Ca}^{++}$  content of synaptosomal membranes as opposed to the lowered content seen after a single dose of ethanol.

**MECHANISMS BY WHICH ETHANOL MAY ALTER NE TURNOVER--ROLE OF TIQ ALKALOIDS**

There are, however, several other phenomena which may occur during the chronic intake of ethanol and contribute to the observed increase in NE turnover. One of the widely debated (48,49) possibilities is the formation of tetrahydroisoquinoline alkaloids (TIQ's) in the CNS during ethanol intoxication. These alkaloids have been demonstrated to arise from the spontaneous condensation of either acetaldehyde (eg. salsolinol) or the aldehyde derivatives of the monoamines (eg. tetrahydropapaveroline) with the neuroamine transmitters. Although these condensation products are isolated in quantity from *in vitro* incubation systems (50), only two reports (51,52) have appeared on the isolation of products resembling the TIQ's from brain of animals receiving ethanol. In one report (51), the formation of salsolinol was found to be dependent on the concomitant administration of pyrogallol. Pyrogallol was shown to act not only as a catechol-o-methyl transferase inhibitor but also as an inhibitor of aldehyde dehydrogenase (53). Inhibition of aldehyde dehydrogenase led to an increase in circulating acetaldehyde when rats received both ethanol and pyrogallol and such increased acetaldehyde levels may have contributed to the formation of salsolinol in these animals (54). Turner et al. (52) have shown that tetrahydropapaveroline can also be isolated from brain, but ethanol administration did not increase the brain levels of this alkaloid. Since the presence of acetaldehyde in brain is a prerequisite for the formation of the TIQ alkaloids, it is of interest that animals injected with ethanol were found to have little or no acetaldehyde present in brain unless blood acetaldehyde levels were particularly high (55,56). It has been postulated that aldehyde dehydrogenase in brain quickly metabolizes most incoming acetaldehyde (55). One has to, however, consider that although brain may have a high capacity for metabolizing acetaldehyde (56), it is precisely such metabolism which would compete (57) for the normal paths of disposition of biogenic aldehydes and lead to formation of TIQ's such as tetrahydropapaveroline and other aberrant reactions (58).

Chronic ethanol administration may, in addition, alter the normal capacity of tissues to metabolize acetaldehyde. Korsten et al. (59) have noted that alcoholics have significantly higher blood acetaldehyde levels than non-alcoholics after receiving equivalent amounts of ethanol. We have also noted (Anderson, Ritzmann and Tabakoff, in preparation) that mice chronically imbibing ethanol are found to have higher circulating levels of acetaldehyde during the later phases of the period of ethanol consumption. Increased blood acetaldehyde levels, if reflected in brain, could lead to increased formation of the TIQ alkaloids.

Thus, although ethanol-dependent formation of TIQ's has yet to be unequivocally demonstrated, one should consider that these compounds may form more readily during chronic ethanol ingestion. These alkaloids have diverse pharmacologic properties, acting as MAO inhibitors (60), catecholamine uptake blockers (61), and releasers of stored catecholamines (62). The TIQ's, particularly the tetrahydropapaverolines, have also been shown to have agonist properties on beta-adrenergic receptors (63). The interaction of the TIQ's with postsynaptic receptors (assuming that they are only weak agonists) could induce activity in a feedback loop which would in turn activate synthesis. On the other hand, the release of TIQ derivatives into the synaptic cleft could result in an interaction with presynaptic alpha receptors, which have been shown by several groups to control the release of NE into the cleft (9,64). Blockade of these alpha receptors would result in overflow release of NE from presynaptic terminals. The depletion of presynaptic stores would also activate amine synthesis.

Alterations in receptor responsiveness to catecholamines in the presence of TIQ alkaloids, and alterations in tyrosine hydroxylase activity which are dependent on changes in the intracellular distribution of  $Ca^{++}$  in the presence of ethanol, could, I feel, provide the explanation for the increased turnover of NE noted after the chronic ethanol consumption.

#### NEUROTRANSMITTER INVOLVEMENT IN THE DEVELOPMENT OF TOLERANCE AND DEPENDENCE

At this point I would like to return and attempt to answer the questions posed above as to whether the increase NE turnover during withdrawal is 1) responsible for the symptoms of withdrawal, 2) a response to the stress of withdrawal, or 3) more related to the manifestations of tolerance to ethanol. One finds that two types of published experiments related to such questions lead to different answers. The time course of the increase in NE turnover as shown in Table 4, demonstrates the presence of the increased turnover at times when no withdrawal signs are present. Such data indicates that increased NE turnover is not simply a protective response to the stress of withdrawal. On the other hand, several authors (65,66) have shown that increased noradrenergic activity in the CNS protects animals from drug, electrically and sound-induced seizures and three sets of authors (21,22,67) have demonstrated that blocking noradrenergic systems and depletion NE potentiates the hyperreactivity and seizures seen during withdrawal. Thus the questions of the exact role of NE in determining withdrawal symptomatology remains unanswered.

Some current results from laboratory (68), however, indicate that an intimate relationship exists between the noradrenergic system and the development of tolerance to ethanol after chronic treatment. We placed control mice and mice pretreated with 6-hydroxydopamine (6-OHDA) in a situation in which they were provided with only one source of food and water, and this source was a liquid diet containing seven percent ethanol. Prior to being placed on this diet and at various intervals after being placed on the diet, the mice were injected with a 3 g/kg challenge dose of ethanol and their temperatures and behavioral responses were monitored. We found that control animals consuming the ethanol-containing diet became quite tolerant to the effects of ethanol, while the animals treated with 6-OHDA prior to chronic ethanol administration did not develop any tolerance to the temperature-lowering or hypnotic effects of ethanol (see Figure 1). On the other hand, no difference in the withdrawal symptomatology was evident between the 6-OHDA-treated animals and the control physically dependent animals (68), thus indicating that 6-OHDA did not affect the development of physical dependence but did affect the development of tolerance.

A fruitful model for the development of tolerance may be the following: an acute dose of ethanol, possibly by depressing transport systems in the choroid plexus, lowers brain  $\text{Ca}^{++}$  levels. This in turn decreases tyrosine hydroxylase activity which coupled with decreased impulse flow would result in a decreased amount of NE in the synaptic cleft. The hypothermia and behavioral depression concomitant with acute ethanol ingestion may be directly or secondarily coupled to this noradrenergic deficit.

On chronic ethanol administration, a tolerance develops to the  $\text{Ca}^{++}$ -lowering effect of ethanol, and in fact  $\text{Ca}^{++}$  levels were found to be increased in synaptic areas of brain (47). This increase in  $\text{Ca}^{++}$  could result in increased tyrosine hydroxylase activity leading to increase in NE available for release with impulse flow and thus result in a tolerance to the depressant effects of ethanol.

Our studies described above also indicate that tolerance and dependence may not be part of a unitary biochemical phenomenon. I do not, however, feel that a single transmitter is responsible for all the concomitants of tolerance and another for physical dependence. Interaction between noradrenergic and serotonergic (69) and noradrenergic and cholinergic (70) systems has been demonstrated on both biochemical and behavioral levels. Thus, a more complete neurochemical profile of tolerant and addicted animals and humans will be invaluable in further explaining the total gamut of symptoms resulting from chronic ethanol ingestion.



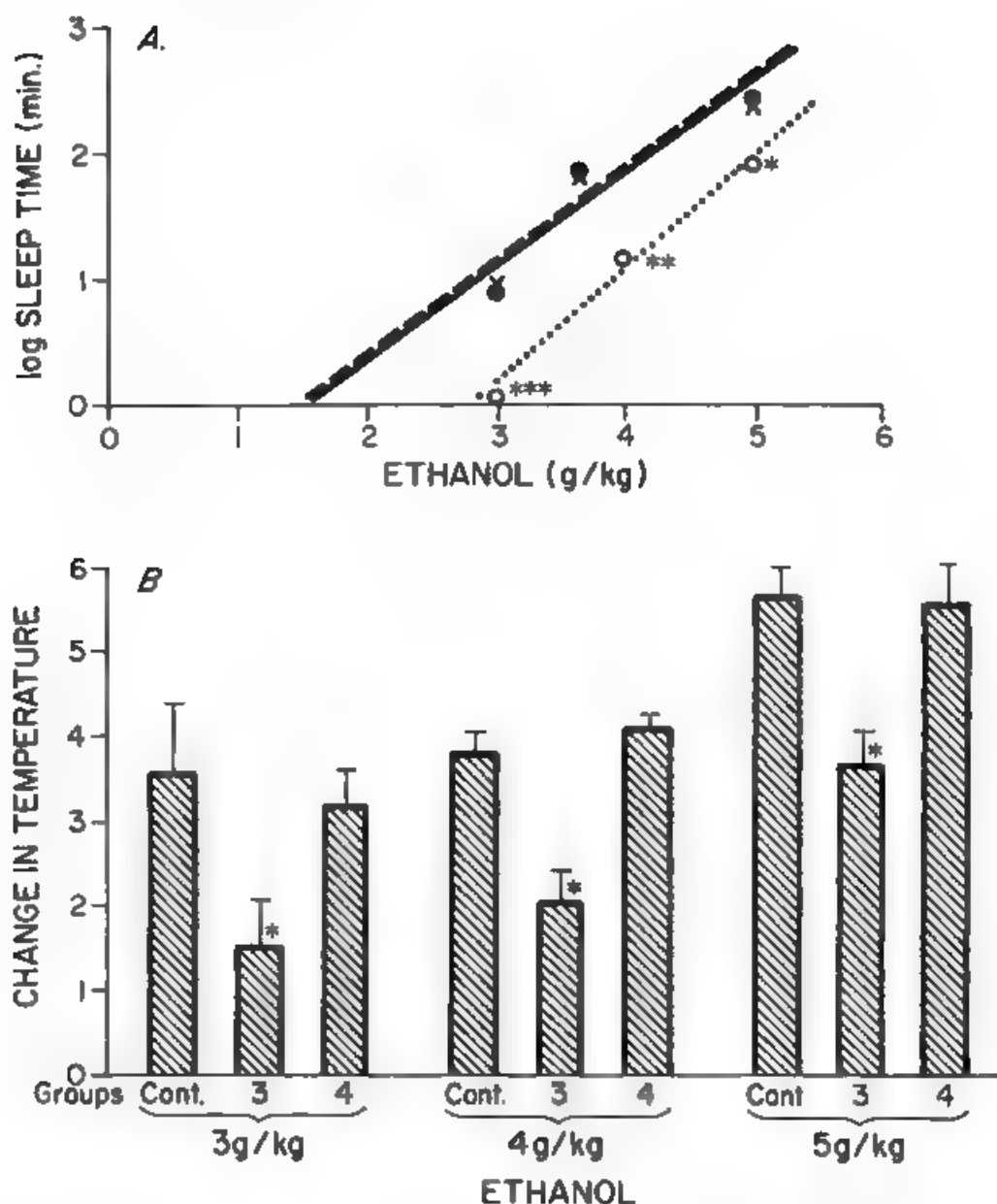


Fig. 1. Mice were injected intraperitoneally with ethanol (3, 4 and 5 g/kg) twenty-six hours after withdrawal, and sleep time (time from loss of righting reflex to time that mice can right themselves twice within a thirty-second period) (A) and body temperature (B) were monitored. Since no differences in these parameters or blood ethanol levels were found between animals in Group 1 (control diet) and Group 2 (6-OHDA and control diet), the values obtained with these animals were pooled and are labelled "control". Animals in Group 3 consumed the diet containing seven percent ethanol and those in Group 4 received 6-OHDA and the ethanol-containing diet.

Insert A--mean sleep times (minutes) in control (0-0), Group 3 (0-0) and Group 4 (X-X) animals after ethanol injection. Statistical comparisons at each dose were made using Student t-test: \*-p < 0.05; \*\*-p < 0.01; \*\*\*-p < 0.001, comparison between Group 3 and Group 4 animals. No significant differences were found between Group 4 and control animals.

Insert B--Rectal temperature was monitored at fifteen and thirty minute intervals for three hours after ethanol injection. The bars represent the mean  $\pm$  S.D. of the greatest drop ( $^{\circ}$ C) from preinjection temperatures recorded during this period. The nadir for the 3 and 4 g/kg dose occurred at thirty minutes and for the 5 g/kg dose at sixty minutes after injection. Comparison between groups at each dose was made using the t-test and starred (\*) groups were significantly different from each of the other groups (p < 0.01) while the unstarred groups were not significantly (p > 0.2) different from one another.

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### 3. ACTIONS OF ETHANOL ON NEURONAL MEMBRANE PROPERTIES AND SYNAPTIC TRANSMISSION\*

Donald S. Faber and Manfred R. Klee

Research Institute on Alcoholism, 1021 Main Street, Buffalo, New York, 14203 and Max Planck Institute for Brain Research, 6 Frankfurt/M-Niederrad, Deutschardenstr. 46, West Germany.

#### INTRODUCTION

Numerous investigations have revealed effects of ethanol on both neuronal excitability and synaptic transmission (cf., 1 for review). Since these studies have utilized a variety of invertebrate and vertebrate model systems and the concentrations employed have often been beyond those associated with moderate intoxication or ataxia, it is not surprising that a myriad of actions have been described for ethanol and that various mechanisms have been postulated to underlie its effects. Ideally, one would like to correlate actions of ethanol on single neurons and neuronal networks with stereotyped changes in behavior. However, while psychophysical tests indicate the vertebrate central nervous system is most sensitive to ethanol (1), the majority of the neurophysiological studies have rather utilized peripheral vertebrate or isolated invertebrate preparations. Nevertheless, these investigations have yielded a great deal of neurophysiological information about the cellular mechanisms of action of ethanol. Such mechanisms generally fall in one of the following categories: 1) a specific reduction in neuronal excitability through alterations in the voltage dependent ionic conductances underlying action potential generation (3-6); 2) indirect effects on excitability consequent to changes in passive membrane permeabilities and resting membrane potential (3,4,7,8,9); and 3) pre- or post-synaptic changes in the efficacy of synaptic transmission, e.g. altered transmitter release or a change in the transmitter

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sensitivity of the post-synaptic membrane-bound receptors (10-18).

In the past few years we have analyzed the actions of ethanol in two model neural systems (4,12,15,19) both of which provide the opportunity to obtain stable intracellular recordings from identified neurons. The first is the abdominal ganglia of the marine mollusk *Aplysia californica*. This preparation is ideal for determining drug effects on membrane potential and resistance and spike electrogenesis, the latter being the membrane property altered most consistently by ethanol in different nervous systems. The results obtained from this preparation are interesting not only in elucidating mechanisms of action but also in emphasizing the fact that neurons are not all alike and have different reactions to ethanol. Presumably, such variations underlie the difficulties in establishing both the specific actions of ethanol and its selectivity within the central nervous system. More recently, we have studied its effects on the goldfish medullary network involving the Mauthner cell (M-cell). The results obtained from this preparation can be correlated with changes in a relatively simple behavior mediated by the M-cell, the startle reflex. Furthermore, there is a striking correspondence between the minimal brain ethanol concentrations necessary to alter the functional organization of this network on the one hand and the behavior of the fish on the other hand (15,19). That is, a relatively specific neuronal effect can be demonstrated at a "physiological" dose level, and additional effects occurring at higher brain ethanol levels can be evaluated in terms of progressive changes in behavior. In this paper, we review and compare the basic actions of ethanol as revealed by these two preparations, in order to develop a more comprehensive understanding of its effects on central nervous system networks.

#### THE INVERTEBRATE SINGLE-CELL APPROACH

The experiments presented here involved intracellular recordings from large neurons in the isolated visceral and pleural ganglia of the marine mollusk *Aplysia californica*. These nerve cells have diameters between 100 and 800  $\mu\text{m}$  and the locations and characteristics of about thirty identifiable cells in the abdominal ganglion have been described extensively (20). Cells can be further distinguished on the basis of their responses to putative neurotransmitters (20-25), their spontaneous activity and endogenous rhythms (20) and their electrogenic properties (26-30). Therefore, precise investigations of a specific cell or group are possible, as are comparative studies within the same ganglion.

Because of the size and unipolar structure of the cells,

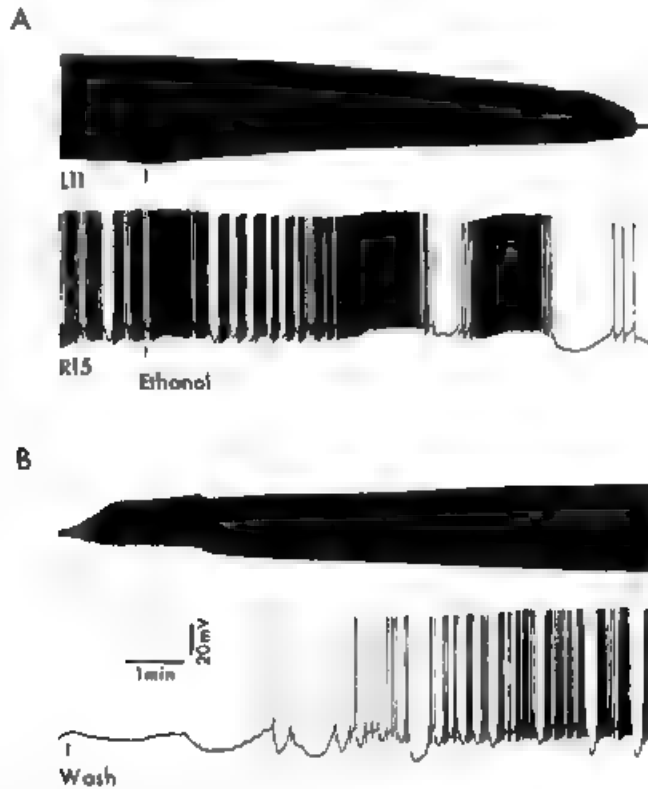
not only voltage recording but also current and voltage clamp techniques can easily be used in this preparation. Voltage clamping allows the direct study of the conductance changes and current flows underlying action potential generation at the level of the cell soma, and the control of this process. Furthermore, characteristics of synaptic transmission can be studied both by stimulating presynaptic inputs and by analyzing the responses of single cells to iontophoretic applications of transmitter candidates. By combining these different approaches the actions of drugs such as ethanol on a broad spectrum of membrane properties can be specified.

### Effects on Membrane Potential and Resistance

Effects of ethanol on the resting membrane potential (RMP) of *Aplysia* neurons were variable, different effects occurring in different cell groups (4,12). Ethanol depolarized one class of cells, hyperpolarized a second, and had no significant effect on the RMP of a third! Within each cell group, moreover, the results were consistent. As will be discussed below these different cell groups have other distinguishing membrane characteristics as well. All the effects of ethanol described in this and the following sections were concentration-related and were completely reversible.

The magnitude of the depolarizations produced by 4% ethanol ranged from 5 to 20 mV. The strongest depolarizations were in cell L-11 (Figure 1), which characteristically had a RMP of around -30 to -40 mV. Within the initial ten minutes of perfusion with 4% ethanol the RMP of this cell was reduced by about 50% and no spikes were generated. In other cells which were consistently depolarized the size of the potential shift was less and it did not result in an inactivation of the spike generating mechanism. In fact, despite these depolarizations most of the cells were less excitable and an increase in the threshold for spike initiation was found with both orthodromic and antidromic stimulations and with transmembrane current injections.

Ethanol-induced depolarizations have also been described for frog muscle fibers, and an increase of resting sodium permeability was postulated by Knutsson and Katz (9) as the mechanism underlying this action. We have directly confirmed this hypothesis by comparing the effects of ethanol in the standard artificial sea water (ASW) perfusing the ganglion and in sodium-free sea water. In four cells which were depolarized an average of 12 mV (range 8-17 mV) by 4% ethanol, perfusion with  $\text{Na}^+$  free ASW resulted in a membrane hyperpolarization which averaged 15 mV (range 7-24 mV). This somewhat large increase in RMP agrees with the relatively high  $P_{\text{Na}}/P_{\text{K}}$  ratio reported by Carpenter (31) for these neurons. In three



**Fig. 1.** Differential effects of ethanol on resting membrane potential of *Aplysia* neurons. **A,B:** simultaneous intracellular recordings were obtained from cells L-11 (upper records) and R-15 (lower records). **A:** ethanol (4%) was added to the ASW at the arrow and shortly thereafter L-11 was depolarized until finally the spike generating mechanism was inactivated. In contrast, R-15 hyperpolarized and the membrane potential oscillations underlying its bursting behavior ceased. **B:** after 15 minutes total exposure to ethanol, the perfusion fluid was switched back to ASW at the arrow. Within minutes the cells began to recover from the effects of ethanol.

of the four cells, the depolarization produced by ethanol was completely blocked in the sodium-free solution. In the fourth cell ethanol produced the same magnitude depolarization in the sodium deficient ASW as in normal ASW, but the absolute membrane potential was 24 mV greater in the former than in the latter. These results are, therefore, consistent with the hypothesis that ethanol increased resting  $P_{Na}$ .

In contrast with the above results another class of cells that was consistently hyperpolarized by ethanol in the concentration range of 2 to 4% could be identified (Figure 1). These hyperpolarizations were dose dependent and reached a maximum of 12 mV. Figure 1 illustrates this effect on the bursting pacemaker cell R-15 (20,32). The membrane potential

of this cell normally oscillates with a depolarizing phase triggering a train of spikes and in turn being followed by a hyperpolarizing wave. In 4% ethanol the oscillations were dampened, and the membrane was hyperpolarized by about 5 to 10 mV. These cells could also be distinguished by the fact that they are all depolarized by acetylcholine (ACh) in contrast to the previous group of cells which are inhibited by ACh.

The alterations in membrane potential produced by ethanol were not associated with any significant changes in membrane resistance, which is in contrast to the small increases in resting membrane conductance reported for squid giant axon (3) and frog muscle fibers (8). Generally, effects of ethanol on membrane resistance are not striking and probably do not contribute significantly to its actions.

#### Effects on Action Potential Generation

In all *Aplysia* neurons investigated, ethanol (2 to 5%) produced reductions in spike amplitude of 20 to 50% (Figure 2 and references 4 and 12). This effect was mainly due to a decreased rate of rise of the action potential. Since the inward current underlying spike initiation in molluscan neurons is carried by both  $\text{Na}^+$  and  $\text{Ca}^{++}$ , (29,30) we tested the possibility that both conductance mechanisms are altered by ethanol. This was the case; ethanol reduces the size of action potentials remaining in either sodium-free or calcium deficient ASW. Furthermore, when the sodium channels were specifically blocked by the addition of 30  $\mu\text{M}$  tetrodotoxin (TTX) to the ASW, 4% ethanol reduced the TTX-insensitive component of the spike by the same amount as in sodium-free ASW. That is, ethanol appears to block both the sodium and calcium components of the action potential.

Voltage clamp experiments are required to discriminate quantitatively the effects of ethanol on the different ionic currents underlying spike electrogenesis. Previous experiments using squid giant axon had produced conflicting results; Armstrong and Binstock (3) reported that ethanol reduced on the transient voltage-dependent increase in sodium conductance underlying spike initiation, while Moore et al., (6) found a similar reduction in the delayed potassium current involved in membrane repolarization as well. Our *Aplysia* experiments were undertaken to resolve this difference and to determine the effects of ethanol on the early inward calcium current not seen in the squid axon. As shown in Figure 3, these studies revealed a relatively specific effect of ethanol on the early inward current. In addition, by altering the  $\text{Na}^+$  and  $\text{Ca}^{++}$  concentrations in the ASW, it was possible to demonstrate that both components of this current were equally

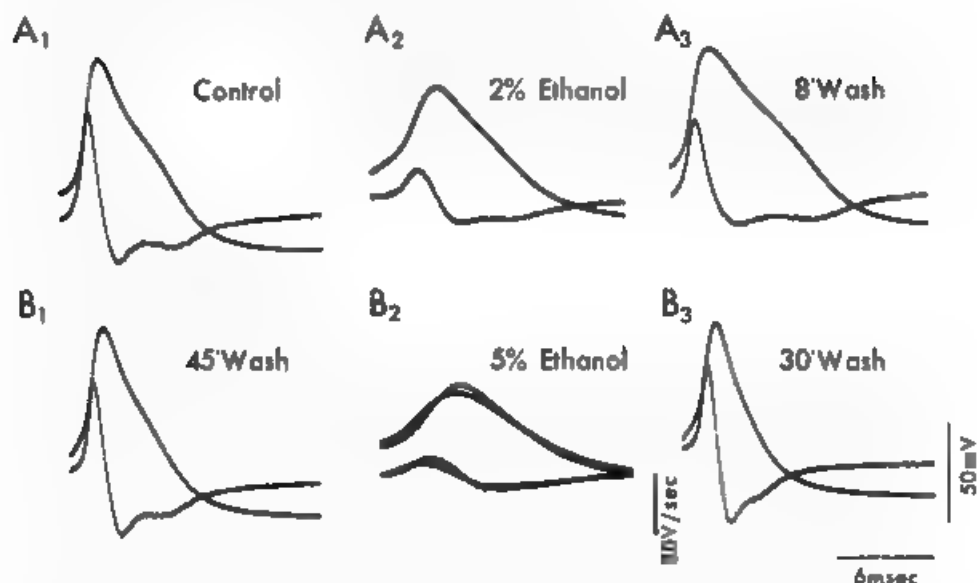


Fig. 2. Effects of ethanol on the action potential of the giant cell in the left pleural ganglion. In all records the simultaneously recorded action potential (upper traces) and its electrically differentiated representation (lower traces) are shown. A1: control recordings. A2: after 10 min exposure to 2% ethanol, spike amplitude decreased and its duration increased. A3 and B1: progressive recovery of the spike after washing in ethanol-free ASW. B2: the effects of ethanol on spike generation were more pronounced when its concentration was raised to 5%. B3: again, washing the preparation with ASW reversed the effects of ethanol.

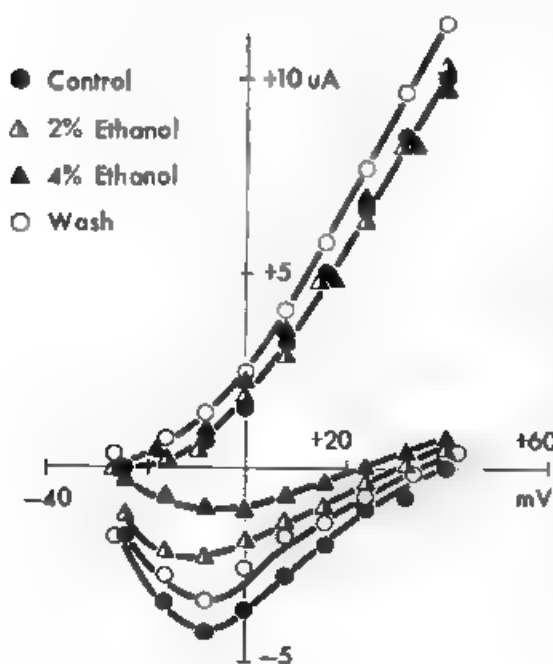
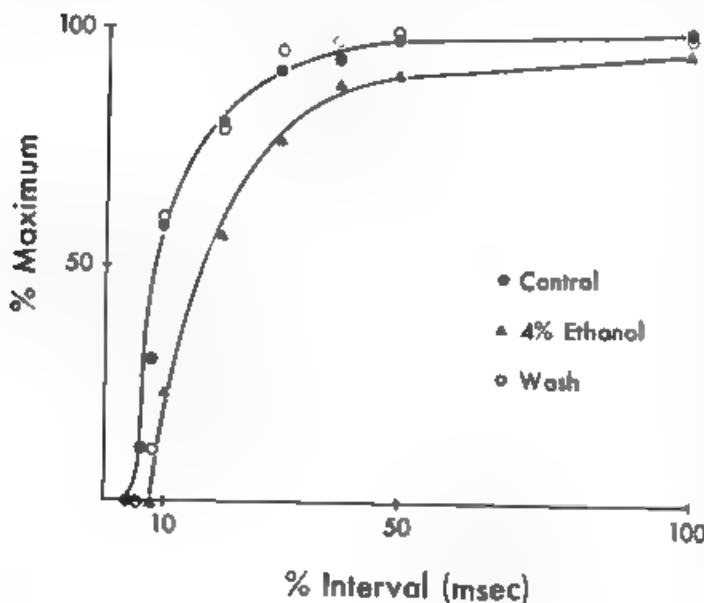


Fig. 3. The effect of two doses of ethanol on the  $I/V$  relationship obtained during voltage clamp experiments. Dose-dependent reduction of the inward current system by 2 and 4% ethanol and unchanged amplitude of the outward current in the giant cell R-2. Abscissa: absolute membrane potential; holding potential was 40 mV. Ordinate: current, outward current is positive (from reference 4).

blocked by 2 to 4% ethanol.

These results confirm the conclusion drawn from the effects of ethanol on spike amplitude and are consistent with the decreased neuronal excitability seen in many systems after ethanol application. Furthermore, since the sodium and calcium currents can be dissected pharmacologically and presumably involve different membrane channels (30), the evidence that ethanol blocks both channels may speak against a specific interaction with the membrane gates controlling channel activation. Rather, it seems reasonable to suggest that it produces a change in membrane structure which consequently alters the characteristics of the channels. In this context the membrane expansion theory of Seeman (33,34) and others merits consideration. The essence of this theory is that anesthetics such as ethanol adsorb to hydrophobic regions of excitable membranes, thereby expanding these regions of membrane proteins and distorting the ionic conductance channels. This theory, however, does not explain why some channels are altered and others such as the voltage dependent  $K^+$  ones, are not.

Experiments on the kinetic properties of the early inward currents revealed an additional significant action of ethanol. It prolonged the recovery of this system after a conditioning stimulus which evoked a maximal increase in  $Na^+$  and  $Ca^{++}$  conductance (Figure 4). In terms of membrane excitability, this observation implies an increase in the relative refractory period and reduction in the maximal firing rate of the neuron. This finding is consistent with recent experiments on cat spinal motoneurons in which one



*Fig. 4. Ethanol slows the recovery of the inward current system from the inactivation produced by a brief conditioning depolarization. The interval between conditioning and test depolarizing pulses was varied from 2 to 100 msec. Ordinate: inward current evoked by the test pulse expressed as a fraction of the maximum current. Abscissa: interval between conditioning and test pulses (4).*

pronounced effect of ethanol was to increase the critical interval at which the cell could follow paired antidromic stimuli (Figure 5; reference 35). Other changes produced by doses of 200-500 mg/kg body weight include membrane hyperpolarizations of 2-10 mV and a slight reduction in spike overshoot. The latter is probably due to the hyperpolarization rather than to a specific action on the voltage-dependent  $\text{Na}^+$  channels since there is no effect on the rising phase of the action potential. At appreciably higher dose levels reductions in both mono- and polysynaptic excitatory postsynaptic potentials (EPSPs) are also seen, which is in agreement with previous reports (36,37).

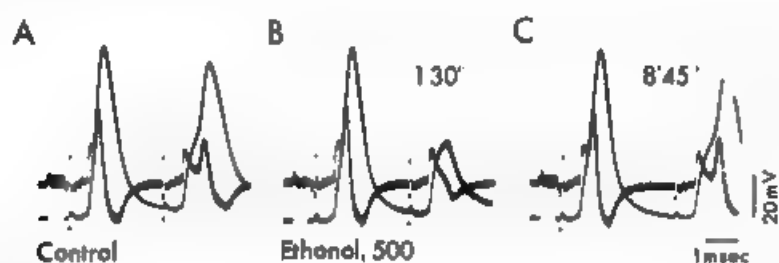


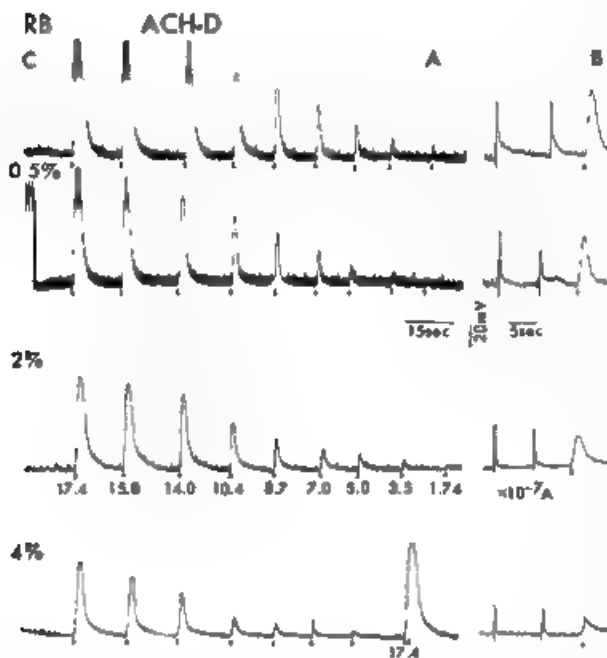
Fig. 5. Increase in the critical interval of cat motoneurons after ethanol injections and impaired spike transmission from the initial segment (IS) to the somadendritic (SD) membrane. A-C: intracellular recordings from a motoneuron. Paired antidromic stimuli were used. A: control responses; stimuli were at the critical interval such that the IS-SD delay was increased for the second response. B: 90 sec. after an i.v. ethanol injection (500 mg/kg), the SD component of the second spike failed. Same stimulus interval as in A. C: 8'45" after the ethanol injection the critical interval is still increased by 20%. In all records voltage responses and their electrically differentiated representations are superimposed.

#### Effects on Synaptic Transmission

Barker (10) has proposed a comprehensive theory for the action of anesthetics based on his observations that in various invertebrate preparations all anesthetics tested (including ethanol) specifically reduced EPSPs involving increased postsynaptic cation conductances. All other PSPs remained unchanged. A similar conclusion was reached by Chase (13) who found ethanol depressed EPSPs in another mollusk, but he apparently did not study its effects on inhibitory PSPs (IPSPs). In our experiments (Bergmann et al., in preparation) blocking effects on synaptic transmission have been observed with ethanol concentrations (0.5-4.0%) appreciably lower than those necessary to alter membrane potential and spike



electrogenesis. However, we have not found the specificity described by Barker. To the contrary, ethanol blocks both excitatory and inhibitory postsynaptic responses (Figures 6 and 7, column B). Furthermore, we have demonstrated that these effects are mediated postsynaptically by investigating the responses of these neurons to iontophoretically applied ACh. Aplysia neurons have been classified as ACh-D- and H-cells depending upon whether they are depolarized or hyperpolarized by ACh, and there are both different receptors mediating these responses and different ionic conductances involved. Specifically, D- responses are due to an increase in membrane conductance to either  $\text{Na}^+$  and  $\text{Ca}^{++}$  ions or to  $\text{Cl}^-$  ions, while H- responses can be either  $\text{Cl}^-$  or  $\text{K}^+$  dependent. The fact that there are  $\text{Cl}^-$  dependent D- and H- responses reflects variations in the  $\text{Cl}^-$  equilibrium potential relative to resting membrane potential. Despite this evidence for different ACh receptors, we have found that ethanol blocks most ACh responses tested in a dose-dependent manner, as is illustrated in Figures 6 and 7, column A, for one excitatory and two inhibitory responses.



**Fig. 6. Reduction of ACh-D-responses and EPSPs by ethanol on a RB neuron.** In each line depolarizing responses to iontophoretic applications of ACh ( $\Delta$ ) with decreasing currents ( $17.4$  to  $1.74 \times 10^{-7}$  A) are shown on the left (column A) and EPSPs are shown on the right (column B). From top to bottom control responses (C) and responses recorded during perfusion with 0.5, 2.0 and 4.0% ethanol are shown. As shown in the lower record the blocking action can be overcome with a double application of the maximal ACh stimulus.

Not all PSPs are blocked by ethanol, and we have found some EPSPs which are extremely resistant to its action. In fact, Woodson et al., (18) have recently focused on one such EPSP and have demonstrated that the only detectable action of ethanol is to accelerate the decay of post-tetanic potentiation. Therefore, it appears that Barker's hypothesis is untenable and that ethanol has selective effects on both

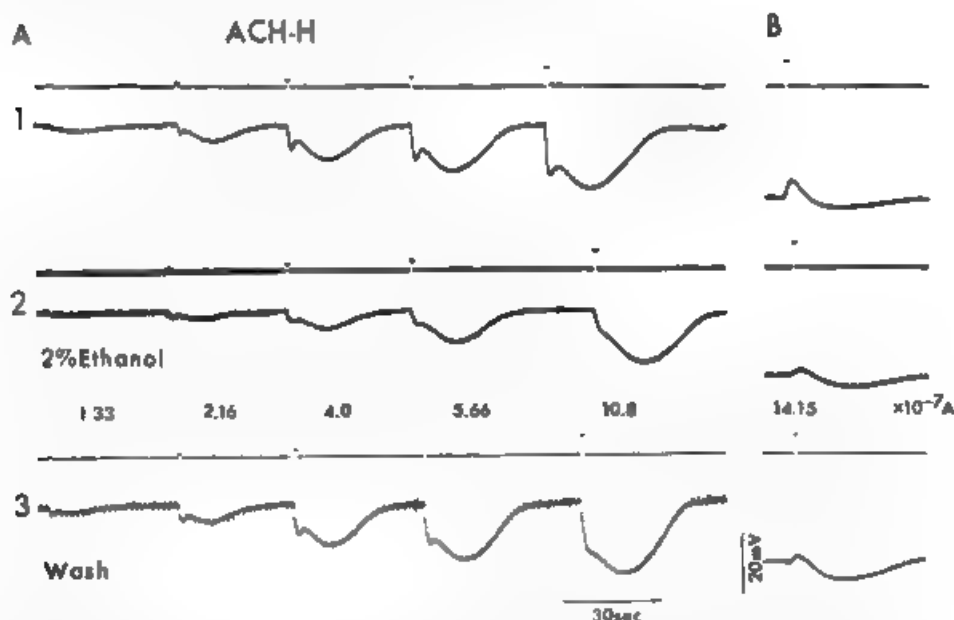


Fig. 7. Ethanol reduces the  $\text{Cl}^-$  and  $\text{K}^+$  components of the ACh-H-responses of a medial cell in the pleural ganglion. A1: with increasing intensities of ACh iontophoresis a two-component H-response is recorded at a resting potential of  $-40$  mV. B: when resting potential is shifted to  $-70$  mV, the early  $\text{Cl}^-$  component is reversed to a depolarizing response while the late  $\text{K}^+$  component is still seen as a hyperpolarization. A2, B2: 2% ethanol reduces both components, its effect on the early  $\text{Cl}^-$  component being stronger. A3, B3: recovery after 30' washing in ethanol-free ASW.

EPSPs and IPSPs. Finally, it should be pointed out that these effects of ethanol on cholinergic responses are the opposite of those generally described for sympathetic ganglia (38) and the vertebrate motoneuron junctions with Renshaw cells (39) and motor endplates (16,17), at which ethanol may rather enhance synaptic transmission.

#### EXPERIMENTS UTILIZING THE M-CELL NETWORK AS A CNS MODEL

The Mauthner cells are a pair of neurons found in the medulla of many fish and amphibia. Since the pioneering studies of the late 50's and early 60's (40-43) our knowledge of the physiology and function of this cell and its associated network has expanded greatly, and the preparation now constitutes an ideal model for the study of many important neurobiological problems. Some relevant background information on the goldfish M-cell, which served as our experimental model (Figure 8) is reviewed here. The M-cell is easily recognized morphologically on the basis of its position and relatively large soma (ca 40  $\mu\text{m}$  diameter) and two main dendrites, one

running laterally and the other ventrally. It has the largest and fastest conducting axon in the goldfish spinal cord by a factor of at least 2. Furthermore, as the M-cell antidromic action potential generates a characteristic extracellular negative field which can be as large as 30-50 mV in the vicinity of the cell's axon hillock (Figure 9-A1) and the intracellularly recorded spike is correspondingly smaller (Figure 9-A2), (40), the cell soma and lateral dendrite can be easily identified electrophysiologically.

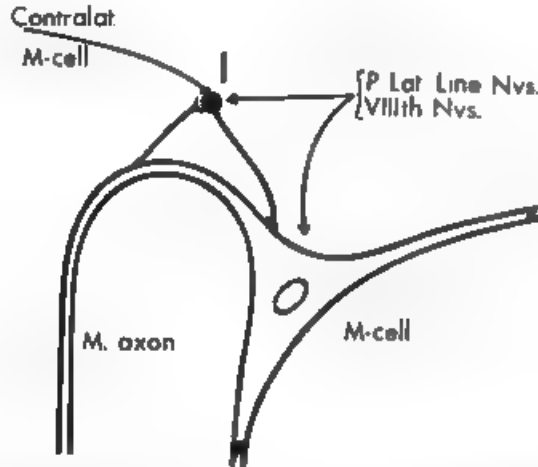


Fig. 8. The schematic model of the M-cell network. The M-cell receives an input from a pool of inhibitory interneurons (I) and excitatory inputs from the VIIIth nerves and both ipsi- and contralateral posterior lateral line nerves (P. lat. line Nvs.). These afferent inputs also project to the inhibitory interneuronal pool, as do axon collaterals from both M-cells.

Afferent excitatory synapses from the ipsilateral vestibular nerve and the posterior lateral line nerves to the lateral dendrites have been extensively studied (44-48), as has the recurrent collateral network mediating somatic inhibition of the M-cell (Figure 8; reference 41, 47-50). Korn and Faber (49,50) have recently identified the interneurons which are activated by M-cell collaterals and in turn feed inhibition back onto the M-cell. They have further shown that the excitatory afferent inputs to the M-cell also converge onto these interneurons (47). Consequently, these cells, which appear functionally similar to spinal Renshaw cells, mediate both collateral and afferent inhibition of the Mauthner neuron. A remarkable feature of this network is the presence of electrical inhibitions mediated by the interneurons and the M-cell. A discussion of these interesting phenomena is not pertinent to this chapter; however, it is important to point out that spike activity in the

inhibitory interneurons produces both electrical and chemical inhibition of the M-cell (51). The former is characterized by an extracellular postivity which is named the EHP and is recorded in the vicinity of the M-cell axon hillock (Figure 9-A1). The EHP then, can be used as a sign of the activation of these neurons, e.g. by M-cell axon collaterals, while the chemical component of the inhibition can be recorded intracellularly from the M-cell either as an IPSP or as an increased membrane conductance (Figure 9-B1). The latter is most easily demonstrated as the reduction in the amplitude of a second antidromic spike when paired spinal stimuli are used (41). It is generally accepted that activation of one Mauthner cell results in contraction of the trunk and tail musculature on the contralateral side of the body through a monosynaptic excitation of spinal motoneurons. The M-cell mediates the goldfish startle or tail flip reflex (42,52,53), which is a basic escape reflex initiated by auditory, lateral line or visual inputs. Since the excitatory transmission from the eighth nerve to the M-cell is so powerful, and there is generally a one-to-one relationship between the firing of the M-cell and that of the spinal cord motoneurons (43) the controls on this reflex are centered in the medulla. Specifically, the control is exerted by the inhibitory network described above (Figure 8). It has therefore been possible to directly compare the effects of ethanol on the neurons in this network with any alterations in its functional output (15,19).

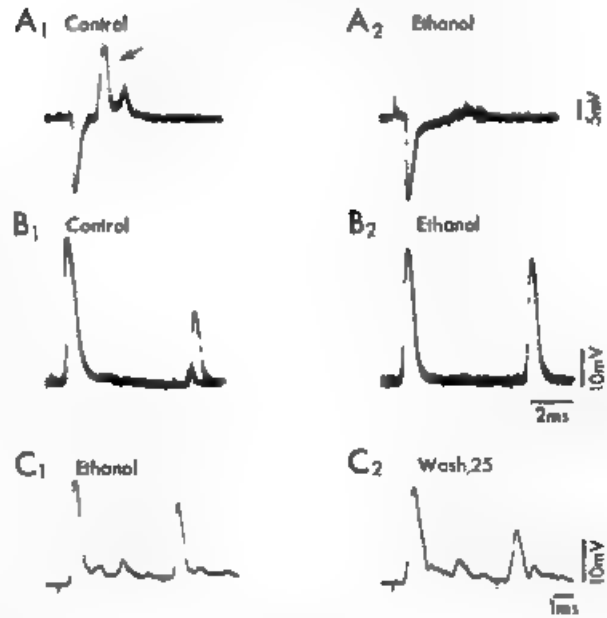
#### Behavioral Effects of Ethanol

Behavioral effects of 1% ethanol (w/v) on freely swimming goldfish were initially determined by gross observations. Brain ethanol concentrations in these fish reached an equilibrium level of approximately 10  $\mu\text{g}/\text{mg}$  brain weight in approximately two hours. During the first thirty to sixty minutes the fish underwent a phase of hyperexcitability characterized by hyperreflexia, including an enhanced startle reflex, poorly coordinated swimming and gulping of air at the water surface. These behavioral effects correlated with brain ethanol levels of 3-5  $\mu\text{g}/\text{mg}$  brain weight. As the ethanol concentration increased, the fish sank to the bottom of the aquarium, became grossly ataxic and overturned, with the loss of their righting reflexes.

#### Effects of M-cell Excitability and Collateral Inhibition

In these experiments ethanol (1 to 2%) was applied through the fluid perfusing the gills and the development of its action was observed during two hours of continuous intracellular

**Fig. 9. Ethanol blocks both the electrical and chemical components of collateral inhibition of the M-cell. In all records the M-cell was activated antidromically by spinal cord stimulation. A<sub>1</sub>, A<sub>2</sub>: extracellular recordings from the axon cap. A<sub>1</sub>: the negative M-cell spike is followed by the positive EHP (arrow) which mediates the electrical inhibition. A<sub>2</sub>: 35 min after starting perfusion with 1% ethanol, the EHP was strongly reduced. Final brain ethanol level**



**20 min later was 4.7  $\mu\text{g}/\text{mg}$  brain wt. B<sub>1</sub>, B<sub>2</sub>: intracellular recordings from the M-cell soma illustrating that ethanol blocks the collateral IPSP as well. B<sub>1</sub>: control. With paired antidromic stimuli the increased conductance associated with the IPSP following the first stimulus either blocks the second spike or reduces its amplitude by about 50%. B<sub>2</sub>: after 60 min perfusion with 1% ethanol this conductance change was almost completely abolished. Brain ethanol concentration was 3.45  $\mu\text{g}/\text{mg}$ . C<sub>1</sub>, C<sub>2</sub>: intracellular recordings from the M-cell in another experiment illustrating the reversibility of the ethanol effect. C<sub>1</sub>: the IPSP was partially blocked during perfusion with 1% ethanol for 45 min. C<sub>2</sub>: the solution perfusing the gills was then switched to an ethanol-free one. 25' later the collateral inhibition was markedly increased. Voltage calibrations in A<sub>2</sub> and B<sub>2</sub> apply to A<sub>1</sub> and B<sub>1</sub>, respectively, the time scale in B<sub>2</sub> for A<sub>1</sub>, A<sub>2</sub> and B<sub>1</sub>, and voltage calibration in C<sub>2</sub> for C<sub>1</sub>.**

recordings from the M-cell. Final brain ethanol concentrations were determined with the techniques of gas chromatography.

Ethanol at a concentration of 3-5  $\mu\text{g}/\text{mg}$  of brain tissue reduces both the electrical (Figure 9-A<sub>1</sub>, -A<sub>2</sub>) and chemical (Figure 9-B<sub>1</sub>, -B<sub>2</sub>) components of the collateral inhibition. Also, as illustrated in Figure 9-C<sub>1</sub>, -C<sub>2</sub> this blockage is reversed when ethanol is removed from the perfusion fluid. In contrast to this action on collateral inhibition, the M-cell resting membrane potential (generally between 70-80 mV) remains unchanged even at levels as high as 20  $\mu\text{g}/\text{mg}$  and only minor changes in its antidromic action potential occurred at the lower ethanol levels. No effects were seen on the

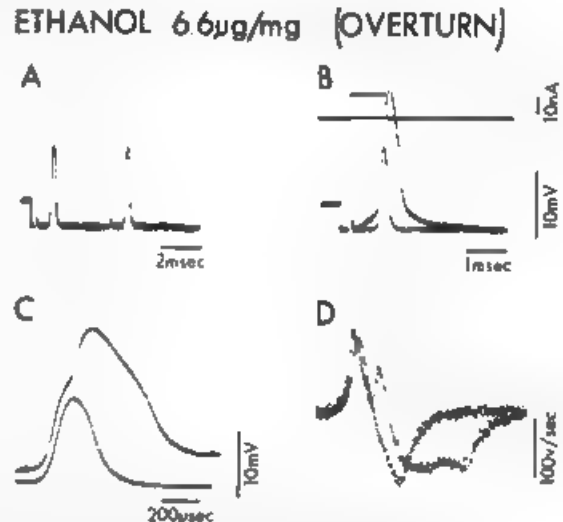
action potential recorded from the M-cell's axon. However, when the brain ethanol concentration was in the range of 6.5-15  $\mu\text{g}/\text{mg}$  brain weight minor effects on excitability were occasionally seen with intracellular recordings at the level of the M-cell soma. Specifically, the safety factor for transmission of the antidromic impulse to the axon hillock was reduced and invasion sometimes failed (Figure 10-A). Under these conditions a full action potential could be restored by pairing a depolarizing current with the antidromic axon spike (Figure 10-B-D), results which suggest an increase in threshold at the axon hillock. This differential effect of ethanol on spike electrogenesis at the axon hillock as opposed to the axon itself is similar to its differential action on the initial segment and some-dendritic membranes of motoneurons. It might be related to a change in the diameter of the involved regions or to basic regional differences in membrane properties.

Fig. 10. Ethanol may reduce the safety factor of spike transmission from the M-cell axon to the axon hillock.

A-D: intracellular M-cell records obtained after a freely-swimming fish reached the behavioral level of "overtake" in 1% ethanol.

Respiration was continued with a 1% ethanol solution during surgery and recordings. A: with antidromic stimuli only a brief axon spike was recorded (paired stimuli were used).

B: when a depolarizing current pulse (upper trace) was paired with the antidromic stimulus the axon spike was then capable of evoking a full-sized M-cell spike (superimposed records with and without the current pulse). C,D: expanded M-cell antidromic action potentials and their electrically differentiated representations, respectively. Superimposed records obtained with and without a depolarizing pulse to facilitate transmission to the axon hillock. Voltage calibration in B pertains for A and time scale in C is for D as well.



#### Site of Action of Ethanol

The blockage of collateral inhibition by ethanol was the only effect observed at the low concentrations. In fact, no other synaptic inputs to the M-cell were reduced by ethanol,

even at higher concentrations. Figure 11 illustrates the fact that neither the excitatory nor inhibitory inputs from the ipsilateral eighth nerve were depressed at concentrations equal to or greater than those which blocked the collateral inhibition. Two sets of findings, therefore, indicate that the depression of collateral inhibition actually occurs at the level of the synapse between the M-cell axon collaterals and the inhibitory interneurons (Figure 9): 1) the blockage of the EHP unambiguously demonstrates a failure of excitation of the interneurons, and 2) the lack of an effect on the

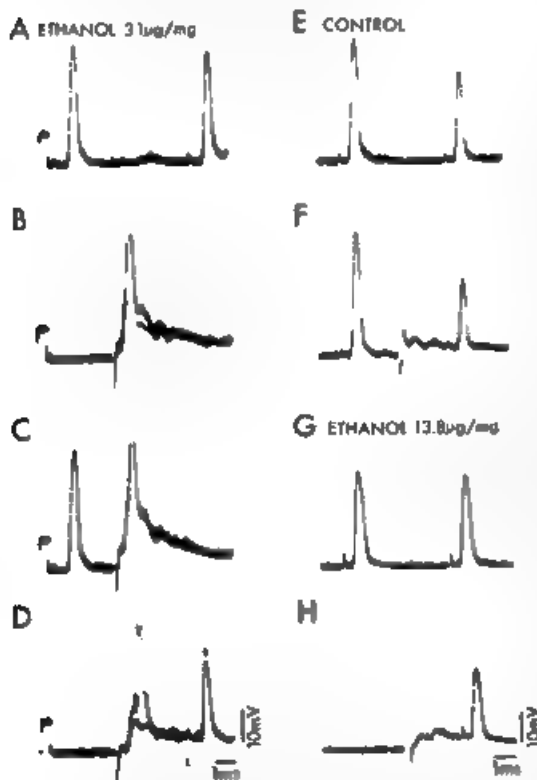


Fig. 11. Evidence that while ethanol blocks collateral inhibition of the M-cell, it does not block afferent inhibitions mediated by the same interneurons. A-D: intracellular M-cell recordings obtained from one experiment during ethanol exposure. A: double antidromic stimuli demonstrate the absence of a collateral IPSP. B,C: stimulation of the ipsilateral VIIIth nerve produced a monosynaptic EPSP suprathreshold for the M-cell (B) and a conditioning antidromic stimulus did not inhibit its effectiveness. D: there is a marked conductance increase when a threshold VIIIth nerve stimulus is used, as indicated by the reduction in the test

antidromic action potential. This is due to the inhibitory input to the M-cell. Final ethanol level was 3.1  $\mu\text{g}/\text{mg}$ . E-H: M-cell intracellular records from another experiment demonstrate that the afferent inhibition is not blocked even at ethanol levels as high as 13.8  $\mu\text{g}/\text{mg}$ . E,F: controls. E: paired antidromic stimuli demonstrating the magnitude of the collateral inhibition. F: interposing a subthreshold VIIIth nerve stimulus between the antidromic stimuli added an additional inhibitory component, as is seen by the further reduction in the test antidromic spike. G-H: records obtained during perfusion with 2% ethanol. The collateral IPSP has disappeared. G: while the VIIIth nerve input still produces a marked inhibition of the M-cell. H: in all traces 2 or more superimposed records are shown; calibrations in D and H also pertain for A-C and E-G, respectively.

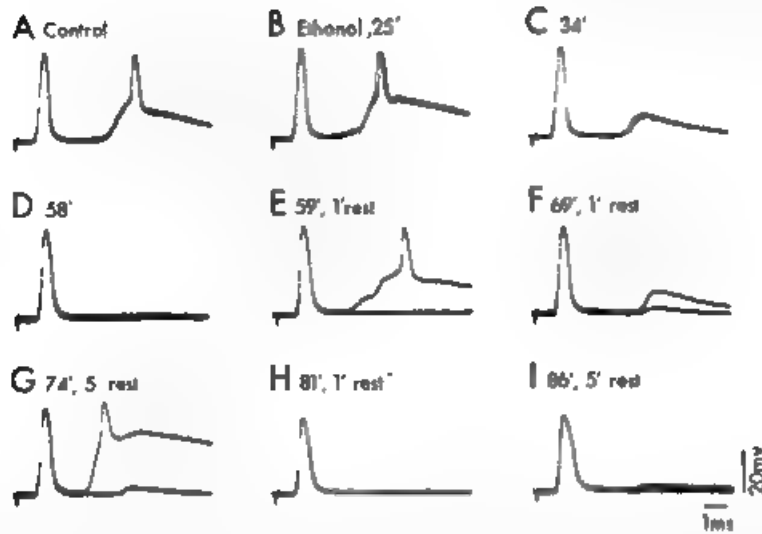
afferent inhibitions mediated by the same interneurons is proof that excitation of the interneurons can still produce an inhibition of the M-cell.

#### Mechanism Underlying Ethanol's Action on Collateral Inhibition

Three possible mechanisms by which ethanol could depress synaptic transmission from M-cell axon collaterals to the inhibitory interneurons have been considered: (1) a presynaptic effect on spike propagation in the axon collaterals, (2) a depression of transmitter release, and (3) a reduction in the sensitivity of the subsynaptic receptors of the inhibitory interneurons to the M-cell transmitter. The first possibility seems unlikely since depression of M-cell excitability only occurred at higher ethanol levels. Nevertheless, a selective effect on the thinner axon collaterals cannot be ruled out. The results of the experiments described below strongly suggest that ethanol acts presynaptically to impair transmitter release. We routinely used a repetition rate of 1/3-1/7 seconds in these experiments. Under these conditions, a stable maximal IPSP can be observed (Figure 12-A; note the IPSP is reversed, i.e. depolarizing, in this figure since a KCl electrode was used for the intracellular recordings). At the lower repetition rate, no effect of 1% ethanol was seen after twenty-five minutes of perfusion (Figure 12-B) but there was typically a 50-70% reduction ten to fifteen minutes later (Figure 12-C). After one hour exposure, the IPSP appeared to be abolished (Figure 12-D). However, we observed at that time a small IPSP could be restored by switching to a lower stimulus frequency and that a full-sized one could be evoked for one stimulus after a one minute period of rest without spinal cord stimulation (Figure 12-E); a second stimulus seven seconds later was completely ineffective (Figure 12-E). As the action of ethanol progressed further, the effectiveness of such one minute rest periods diminished appreciably (Figure 12-F) and longer rest periods in the range of five minutes were necessary (Figure 12-G). Again, however, only the first in a train of stimuli at a 1/7 second repetition rate evoked an IPSP. Finally, after eighty to ninety minutes total perfusion time with ethanol, such rest periods were completely ineffective and the IPSP was completely blocked (Figure 12-H and -I). At that time, the ethanol level in the experiment illustrated in Figure 12 was 4.51  $\mu\text{g}/\text{mg}$  brain weight, and we estimate that it was no more than 2-3  $\mu\text{g}/\text{mg}$  brain weight during the first hour of exposure.

The evidence that such prolonged rest periods are capable of transiently restoring synaptic transmission suggests that ethanol acts on the process of transmitter release from the M-cell axon collaterals, and does not appear readily compatible





**Fig. 12.** Time course of the development of the ethanol effect on the collateral IPSP. The records were obtained with KCL-containing microelectrode. Three or more superimposed traces of the M-cell responses to spinal cord stimulation at a rate of 1/7 seconds are shown in A-I. A: control. The antidromic action potential elicited by a single shock to the M-axon is followed by a depolarizing IPSP which causes the cell to initiate a second spike. B-I: records obtained at the indicated times after starting perfusion with 1% ethanol. The traces illustrated in E, F and H were preceded by one minute rest periods during which no stimulation was employed, and those illustrated in G and I were preceded by five minute rest periods. B-D: gradual reduction and, finally, blockage of the IPSP over a fifty-eight minute period. E: at fifty-nine minute ethanol, a one minute rest restored the IPSP for the first stimulus in a train with a repetition rate of 1/7 seconds. F: diminished effectiveness of the one minute rest ten minutes later. G: at seventy-four minutes, a five minute rest restored the IPSP for one stimulus to a magnitude greater than that in control. H: eighty-one minutes after starting perfusion with ethanol, the one minute rest was completely ineffective and no IPSP was evoked. I: after an additional five minute rest, an IPSP less than 10% the amplitude of that illustrated in control could be evoked by the first stimulus in the 1/7 second train (2).

with the effects on collateral excitability or the sensitivity of the postsynaptic neurons to the M-cell transmitter. This conclusion is consistent with the biochemical studies of Kalant et al., (54), which indicated that incubation with ethanol inhibits acetylcholine release from rat cerebral cortex slices. The correlation is further strengthened by evidence that the transmitter released by the goldfish M-cell

(Faber and Klee, unpublished observations) is ACh, as is also the case with the hatchet fish M-cell (55). It is also interesting that Weakly (56) similarly concluded that barbiturates act presynaptically to depress transmitter release in the mammalian spinal cord.

Our preliminary behavioral observations indicate that the initial phase of hyperexcitability following exposure to ethanol occurs at the same brain ethanol levels which rather selectively depress excitatory synaptic transmission from the M-cell collaterals onto the interneurons exerting a major inhibitory control of the startle reflex. In addition, higher anesthetic levels may be associated with direct effects on excitability as well. Clearly, more quantitative behavioral experiments on the effects of ethanol and additional experiments on the other components of the neural network involved in the startle reflex are needed. Nevertheless, it is clear that this preparation indeed offers a promising approach to the general problem of correlating physiological and behavioral aspects of drug actions.

## DISCUSSION

Comparison of the results obtained with the *Aplysia* and M-cell models provides a framework for some general conclusions concerning the actions of ethanol. One is that the mechanisms of synaptic transmission appear more sensitive to ethanol than either spike electrogenesis or the membrane properties controlling resting membrane potential. This conclusion is based largely on the evidence that cholinergic transmission is depressed in both systems by ethanol concentrations significantly lower than those which alter the other processes. Furthermore, the actions on synaptic transmission are selective and not all PSPs are blocked or reduced by ethanol. We, therefore, suggest that effects on electrical excitability are secondary to those on synaptic transmission. The latter would be primarily responsible for the more specific ethanol effects which are manifested behaviorally as the hyperexcitability, loss of motor coordination, etc., and the former would contribute to its general depressant properties.

Ethanol enhances cholinergic transmission both in the cat spinal cord (motoneurons to Renshaw cells, reference 39) and at the frog neuromuscular junction (16,17). It is not clear why a depression occurs at some cholinergic junctions and a facilitation at others. Possible explanations include regional differences in the characteristics of transmitter synthesis and release and differences in postsynaptic receptors. One interesting experiment would be to determine if the synaptic transmissions mediated in the spinal cord by

M-cell axon collaterals are also blocked by ethanol or if it has different actions on different presynaptic processes of the same cell.

One of the most intriguing aspects of our results is that in both the cat spinal motoneuron and goldfish Mauthner cell ethanol did not produce a generalized depression of spike electrogenesis. Rather the M-cell axon hillock and the motoneuron soma-dendritic membranes were preferentially depressed. This selective action would contribute to delaying or blocking synaptic activation of the neurons and would reduce their maximum firing rates. The mechanism underlying this selectivity is not clear; it may relate to the lack of myelination of these membrane regions or to a lower density of voltage-dependent  $\text{Na}^+$  channels.

The above observations point directly to a situation which neurophysiology has recently come to face. Namely, the membrane properties of neurons differ and there is no standard cell which can be used for neuropharmacological investigations. The opposing effects of ethanol on RMP of different *Aplysia* neurons is another clear demonstration of this problem. It is therefore almost impossible to evaluate the significance of these RMP changes, and the general consequences of such a situation are clear; in order to determine the mechanisms of action of a substance such as ethanol it is first necessary to identify the systems responsible for its behavioral effects. Then, drug action should be studied in this system and concentration levels maintained in that range with which the behavioral change is obtained. In this context, as described above, the M-cell and its associated network offers an ideal model system in which all of the basic tools available can be applied to such problems. It is therefore gratifying that the results agree: behavioral hyperexcitability is correlated with a disinhibition of the M-cell due to a specific effect on one synaptic system, and a more general anesthetic action is correlated with decreased neuronal excitability. In fact, we consider ourselves lucky that ethanol has such a clear action on this system. More often the systems best suited for such a combined approach turn out to be those which do not react to the drug being studied!

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#### 4. TOLERANCE AS ADAPTATION: INTERACTIONS WITH BEHAVIOR AND PARALLELS TO OTHER ADAPTIVE PROCESSES

A. Eugene Le Blanc and Howard Cappell

Addiction Research Foundation, Toronto, Canada.

##### INTRODUCTION

It is clear from even a cursory reading of the vast literature on drug tolerance that approaches to this interesting phenomenon vary widely in basic conception. Whereas some investigators concentrate their efforts upon isolated, albeit fundamental units of biological function, such as neurotransmitters (e.g., 1), others have opted instead for an approach that emphasizes an integrated functional adaptation of entire organisms behaving in a demanding environment (e.g., 2). There is no necessary conflict in these approaches; indeed, any thoughtful observer must see an ultimate connection between these apparently different enterprises. It will become clear that our inclination is to view the organism in the broader context of environmental adaptation; indeed, it is the thesis of this paper that tolerance to drug effects represents a special case of the general adaptive capacity of organisms. Yet this will not be the thesis of apostates who score belief in the events that go on within the venerable "black box"; on the contrary, studies involving manipulations of these events are basic to our conception of tolerance. The strategy will be to draw parallels between adaptations to the behavioral and biologic consequences of drugs and other forms of demand that appear to evoke an adaptive response. Additionally, we will attempt to show how aspects of tolerance to drug effects are subject to environmental control in a way that requires a more complex interpretation of tolerance than changes of receptor sensitivity at the site of a drug's action can alone provide.

The purpose of this paper is to attempt a synthesis. For that reason our strategy will be to look for consistencies despite an awareness that all is not as neat as the analysis may imply. In this regard, it must be admitted from the outset that the parallels to be noted essentially involve the phenomenon of tolerance to the relative exclusion of physical dependence, despite evidence that they are intimately associated developments. Thus it is much more difficult to



find compelling examples of postadaptational supersensitivity when dealing with nonpharmacological rather than pharmacological adaptations. With this hopefully disarming admission of fallibility on record, let us now begin to turn to the issues at hand.

#### *WHAT IS TOLERANCE?*

Since the word tolerance will be used repeatedly, it behooves us to begin with a definition of its descriptive requirements. A widely acceptable definition includes the following requirements: (a) a drug effect will diminish in magnitude with repeated exposure to a fixed dose; and (b) a drug effect diminished in magnitude by repeated exposure to a fixed dose can be reinstated to its original level by increasing the dose.

Although it is possible to define tolerance precisely, use of the term is complicated by the existence of special cases such as "physiological" tolerance, "behavioral" tolerance, and "functional" tolerance. An added difficulty in speaking of tolerance is that it develops at different rates depending on the effect and drug in question, and does not develop at all where some drug effects are concerned. As far as possible, we will attempt to avoid the pitfalls attendant to the linguistic and empirical complications associated with discourses on tolerance phenomena.

Ultimately, any discussion of tolerance is incomplete to the extent that it skirts the issue of the mechanism or mechanisms involved. It seems more than a matter of faith that tolerance is accountable in reductionist terms, and indeed such accounts exist (e.g., 1). Yet not all productive conceptions of tolerance are rooted in a reductionist tradition, as will become evident in our discussion of work containing a large element of behavioral analysis. What remains then, is a consideration of the pertinent literature, with particular emphasis on work conducted in the laboratories of the Addiction Research Foundation.

#### *EMPIRICAL EVIDENCE*

##### Effects of Cortical Ablations

In looking for parallels between tolerance and general adaptive processes, one potentially useful strategy is to compare the impact on these processes of attempts to interfere with them. One such attempt has involved surgical intervention by ablation of the frontal cortex. A considerable body of research (3) indicates that an intact frontal cortex is necessary in several species to acquire behavior requiring a

delay of response or a sensory discrimination in order to receive reward. Thus, frontal cortical ablations may interfere with learning. Moreover, lesions of the frontal cortex have been shown to impair the physiological adaptation to thermally elicited tachycardia that is evident in normal animals (4). If tolerance shares something in common with these other adaptive phenomena, it should be possible to interfere with its acquisition by means of comparable ablations: just such an hypothesis was tested in an experiment by Le Blanc, Matsunaga, and Kalant (5). Rats were first made tolerant to the impairment produced by alcohol on the "moving-belt" test, which provides sensitive measure of motor performance (cf. 6). Tolerance was established over a three week period during which a maximum daily dose of 6 g/kg (by gavage) of ethanol was attained. Tolerance was assessed with periodic test trials in which the impairing effect of 2 g/kg i.p. of ethanol was measured. A period of one month was allowed for recovery before animals were assigned to groups selected for lesions, sham operations, or nonoperated controls. The surgery involved bilateral frontal polar lesions 3 x 5 mm in extent. Following a nine day postoperative recovery period, all animals were once more subjected to the same regime of alcohol exposure as before. Rats that were sham-operated or nonoperated became tolerant to the extent that impairment was reduced by more than 50% over the course of six test trials; lesioned animals, in contrast, displayed virtually no improvement in performance during a similar schedule of exposure to alcohol. A smaller pilot study yielded similar results where lesions to the occipital cortex were concerned. In summary, while much remains to be learned of the basis for this positive result, alcohol tolerance was shown to share a property in common with complex learning and with a fundamental process of physiological adaptation. Other research on amphetamine (7) suggests that the involvement of the frontal cortex in tolerance development is not peculiar to alcohol or to motor impairment.

#### Inhibition of Protein Synthesis

The evidence on the effects of frontal cortical ablations provided some support for a commonality of process in tolerance development and the acquisition of new responses. To the extent that protein synthesis is involved in learning, another opportunity for pursuing the analogy presents itself. Evidence of a role for protein synthesis in learning is provided in a study by Segal, Squire, and Barondes (8), who showed that cycloheximide, an inhibitor of central protein synthesis, interfered with the retention of discrimination learning in mice. What of the effect of cycloheximide on alcohol

tolerance? This question was pursued by Le Blanc et al. (5) using a design and procedures very similar to that adopted in the ablation study; the major difference was that the effects on reacquisition of tolerance after treatment with cycloheximide was at issue. During exposure to the tolerance inducing regime, cycloheximide treatment (0.3 mg/kg) was combined with alcohol gavage in the critical experimental group. On tests of the reacquisition of tolerance to the impairing effects of ethanol, the performance of controls improved over time by more than 50%, whereas rats treated with cycloheximide acquired virtually no tolerance whatsoever. Hence, the evidence for our general hypothesis increases, although it is conceded that this experiment does not rule out mechanisms of interference with tolerance acquisition not involving the inhibition of central protein synthesis (e.g., subclinical convulsions). The effect of cycloheximide on tolerance is not unique to alcohol, since cycloheximide has been found to inhibit the development of tolerance to the analgesic property of morphine in mice (9).

#### Depletion of Serotonin

A third source of evidence for the tolerance/adaptation parallel derives from recent data on the role of serotonin in the development of tolerance to ethanol. That depletion of serotonin retards habituation to non-drug stimuli has been amply demonstrated in studies of acoustic startle responding (10,11,12). In all of these studies, the serotonin level was manipulated by prior treatment with p-chlorophenylalanine (pCPA), an established depletor of serotonin in the CNS. The drug studies (13) employed the moving-belt test to measure tolerance. Both alcohol and pentobarbital were investigated, but since the results were quite similar with each drug only the ethanol work will be presented in any detail. The essential strategy of the study was to expose animals depleted of serotonin to a schedule of alcohol administration that would be expected to promote tolerance to the impairing effects of a test dose of 2.2 g/kg of ethanol. Treatment with pCPA (100 mg/kg/day, i.p.) was given daily for ten days before chronic exposure to alcohol (5.0 g/kg/day p.o. 25% V.V.), and continued for twenty-five further days during which experimental animals were also exposed daily to large doses of alcohol by gavage. Tests of impairment on the moving-belt apparatus were interspersed at intervals during the chronic regime of treatment. Control animals exposed to ethanol, but not pCPA, clearly developed tolerance to the test dose; rats exposed to pCPA also displayed some tolerance, but the rate of acquisition was significantly impaired, and the level did not approach that of controls during the course of the experiment.

Much the same assertion can be made where pentobarbital was concerned.

As with the other data presented in support of our general hypothesis, these findings are by no means free of alternative interpretation. Yet they do seem to enhance the credibility of the hypothesis by providing supporting data from another general domain of investigation. Moreover, as with the previous illustrations in the first two sections of this chapter, there is evidence that comparable manipulations retard the development of tolerance to a pharmacologically distinct compound, namely morphine (15).

### Topographical Similarity Between Tolerance and Learning

A fourth source of parallels between tolerance and adaptation derives from a topographical similarity in one aspect of tolerance and learning. One property of learning that is demonstrable by appealing to both personal experience and the scientific literature (16), is that responses are reacquired after a period of disuse with much greater facility than they are initially mastered. A similar process can be demonstrated in studies of adaptations of a more fundamental physiological nature; for example, physiological adaptation to thermal stimuli proceeds more rapidly to the extent that an organism has a history of adaptation to those stimuli (4). The parallel was nicely confirmed in a study by Kalant, Le Blanc and Gibbins (17), in which repeated cycles of acquisition of tolerance to alcohol were studied using the moving-belt test. Rats were exposed to as many as four cycles of acquisition with seventeen day drug-free intervals between cycles to permit recovery to baseline levels of impairment. The basic finding was that the same level of tolerance was achieved during each cycle, but that this level was attained in fewer and fewer trials over successive cycles of acquisition. Whereas thirteen to sixteen days of chronic exposure were required for maximal tolerance to be attained during an initial cycle, maximal tolerance was evident with four days of treatment during a fourth cycle of tolerance acquisition.

### Summary of "Parallels"

Clearly, we have presented an idealized version of the similarities between tolerance and other forms of adaptive response. Although specifics were not considered, there are alternative interpretations for many of the findings presented in support of the general hypothesis advanced here. Nonetheless, the possibility of genuine parallels seems too strong to be dismissed easily, and the overall weight of the evidence is consistent with the hypothesis. Moreover, the parallels

appear to cover a broad spectrum of pharmacological agents to which tolerance can be demonstrated, including morphine, amphetamine, alcohol, and pentobarbital. It is obvious that more evidence will be required to move this thesis beyond the point of credible speculation, but there is good reason to believe that such evidence will be possible to obtain.

#### *INTERACTIONS BETWEEN BEHAVIORAL MANIPULATIONS AND TOLERANCE DEVELOPMENT*

The previous sections of this paper dealt with direct parallels between tolerance and other forms of adaptation. Now we turn to a different but not entirely independent issue, namely, the modifiability of tolerance by behavioral interventions.

#### Tolerance and Reinforcement

One of the earliest, and still one of the most intriguing demonstrations of the behavioral modifiability of tolerance was reported by Schuster et al. (2), who analysed tolerance to amphetamine as a form of functional adaptation to environmental contingencies. Rats were trained to press a lever on two schedules of reinforcement that required temporal control of responding in order to optimize the receipt of reinforcement in the form of food pellets. When the animals were responding stably on these schedules they were repeatedly injected with d-Amphetamine (1.0 mg/kg) prior to the behavioral sessions. Amphetamine had a clear disruptive effect on response rate compared to control sessions in which only saline was administered. The interesting result occurred in examining the extent to which response rate returned to normal over a course of chronic exposure to amphetamine. For some animals, the disruption in responding was such that it eventuated in a reduction in reinforcements obtained during a test. For others, although response rate was clearly affected, the reinforcement schedule was such that there was no loss of reinforcements despite the alteration in response rate. In the case of animals that did not suffer a loss of reinforcement, the response rate did not return to control levels even over a course of chronic drug treatment. However, when there was a loss of reinforcements as a result of the behavioral disruption, there was a return of responding to a degree that restored the receipt of reinforcements to control levels. Moreover, a second experiment demonstrated a failure to develop tolerance when injections of amphetamine improved the animals' responding to avoid shock. This led the authors to postulate a form of adaptation that they described as "behavioral tolerance": "Behavioral tolerance will develop in those aspects of the organism's behavioral repertoire where

the action of the drug is such that it disrupts the organism's behavior in meeting the environmental requirement for reinforcements. Conversely, where the actions of the drug enhance or do not affect the organism's behavior in meeting reinforcement requirements we do not expect the development of behavioral tolerance." (2)

This fascinating demonstration has had the effect of substantially altering subsequent conceptions of tolerance for many investigators, for it demonstrated that sheer exposure to a drug was not in itself sufficient to provoke tolerance to one of its demonstrable consequences. Rather, the adaptation was functional in the sense that it occurred only to the extent that it was instrumental in restoring to normal or actually enhancing a particular level of reinforcement.

In a study directly derivative from the work of Schuster et al. (2), Carlton and Wolgin (18) examined the acquisition of tolerance to the anorexigenic effect of amphetamine. By varying the temporal relationship between drug administration and exposure to the behavioral test, they were able to hold constant the pharmacologic stimulus while varying the behavioral contingency. Some of their rats received injections (2.0 or 3.0 mg/kg of d-Amphetamine) twenty minutes prior to a test of milk intake, and some received injections only after the drinking test was completed. Over repeated test trials those animals receiving injections before the test recovered to normal levels of milk consumption. During the same period, the milk intake of the rats that received amphetamine after the test was unaffected. When tolerance was clearly evident in the "before" condition, the temporal relationship between injection and test was reversed in the "after" condition. Interestingly, milk consumption was depressed to an extent predictable if their animals *had never before been exposed to amphetamine*; moreover, the rate of acquisition of tolerance was not distinguishable from that of the "before" group, that was, in fact, drug-naïve before tolerance development was assessed. Carlton and Wolgin (18) arrived at essentially the same interpretation as did Schuster et al. (2); namely, the development of tolerance depended upon the drug-induced loss of reinforcement for its occurrence. They labelled this phenomenon "contingent tolerance" a term that is conceptually similar to "behavioral tolerance".

When we earlier proposed a parallel between tolerance and adaptation, the semantic loading of the word "adaptation" did not go beyond description of a relationship between repeated stimulation and a recovery of response; in this research on amphetamine, however, the word takes on a new meaning that is tinged with the notion of purposiveness and benefit to the organism. In other words, the implication from

this research is that tolerance does not develop to certain drug effects unless it is, in a sense, useful to the organism.

### "Behavioral" versus "Physiological" Tolerance

Behavioral tolerance is a discovery of comparatively recent vintage. It is customary to contrast it with the more traditional conception of tolerance that has come to be known as "physiological". The latter term implies a change in the sensitivity of the neurons directly affected by the drug, and should be a consequence of mere exposure; this is to be distinguished from the functional adaptation implied by behavioral tolerance.

One of the earliest systematic studies of this distinction was reported by Chen (19), who used a design not unlike that of Carlton and Wolgin (18). In this instance, however, alcohol was the drug of interest. Chen's behavioral group received injections of alcohol (1.2 g/kg) before each of four trials in a maze task in which approach behavior was maintained by a food reward. A physiologic group received an equivalent injection of alcohol after testing for the first three trials, but before testing on the fourth. As measured by performance on the fourth trial, tolerance was evident in the behavioral but not in the physiologic group. Chen's experiment was criticized on several grounds by Le Blanc et al. (20), but most importantly because the evidence was insufficient to warrant the qualitative distinction between the two varieties of tolerance implied by Chen. Rather, they argued, the difference might simply be one of rate of acquisition; Chen's experimental design did not permit a test of this hypothesis. Le Blanc et al. (20) were able to assess their alternative essentially by extending Chen's procedure to include many more test trials within the context of a generally more complete experimental design. The results were clearcut: although a "behavioral" group developed tolerance much more quickly than a "physiological" group, the performance of the latter eventually improved to the level of the former. It is clear from the data of the study that Chen's conclusions were based on a schedule of testing that was simply terminated prematurely. Le Blanc et al. (20) concluded that if the only difference in these two ostensibly different types of tolerance was one of rate in attaining the same asymptotic levels, there was no need to postulate more than one basic cellular mechanism of tolerance. For this reason, they coined the phrase "behaviorally augmented tolerance" to describe the interaction between behavioral demand and the rate of tolerance development, while negating the suggestion of qualitatively different mechanisms of tolerance. Moreover, other evidence based on findings with

opiates (21), barbiturates (22) and chlorpromazine (23) can be adduced in support of this argument, although one study of morphine (24) could be interpreted as showing a difference in asymptote as well as rate of tolerance development. Unfortunately, in any given study it is impossible to determine whether testing for tolerance development was terminated before genuinely asymptotic levels were attained.

The evidence in favor of a unitary mechanism underlying behavioral and physiologic tolerance was recently extended (25). In an initial phase of this experiment rats were exposed to the standard procedure for contrasting behaviorally augmented and physiologic tolerance (20); a major difference was that the procedures were not drawn out to the point of convergence in tolerance as they were in the earlier study. Using the moving-belt test to measure tolerance, Le Blanc et al. (25) confirmed the expectation that tolerance would develop in a group receiving alcohol before exposure to the behavioral task but not afterward. This result was, of course, not novel; what was compelling, however, was the finding that the behavioral augmentation group was tolerant on a second behavioral task (maze performance) on which performance was empirically shown to be entirely independent of the first (i.e., there was no evidence of transfer of training), but the physiologic group, despite pharmacologically equivalent exposure to alcohol, performed no better than controls who were pharmacologically naive. This finding is of crucial importance because it demonstrates that behaviorally augmented tolerance is more than learned compensation for the impairment produced in a particular behavioral test; such an argument is untenable in view of the fact that prior learning in one task was without facilitating consequences in acquiring the other. Rather, a more tenable argument is that behavioral augmentation facilitated the development of a fundamental adaptation at the neuronal level that is common to all manifestations of tolerance to alcohol. Parenthetically, it is worth mentioning that behavioral augmentation of physical dependence was also shown in this work.

In summary, the hypothesis that currently enjoys the strongest support is that there is but one basic process underlying tolerance phenomena where neuronal mechanisms are concerned. Yet this process is significantly influenced by the adaptive or functional requirements of the organism in the face of drug-produced impairment. This interaction cannot be ignored in any general mechanistic theory of tolerance.

#### **TOLERANCE AS CONDITIONING: A RADICAL VIEW**

It is generally accepted that behavioral conditioning processes are intimately involved in drug dependence. A role



for conditioning has found one of its most persuasive advocates in Wikler (e.g., 26) although his concern has been primarily to account for relapse using the concepts of Pavlovian or classical conditioning. Recently, Siegal (27) showed that Pavlovian conditioning could completely account for tolerance to the analgesic properties of morphine. In Siegal's words: "According to the present conditioning theory, tolerance to the analgesic effects of morphine results because environmental cues regularly paired with the administration of the drug come to elicit a compensatory (conditioned response) hyperalgesia, which algebraically summates with the stable, unconditioned analgesic effects of the narcotic. Thus environmental cues consistently predicting the systemic effects of the drug should be crucial to the development of tolerance since they enable the subject to make timely compensatory (conditioned responses) in anticipation of the analgesic (unconditioned response)." (27).

Siegal began by presenting ample evidence that repeated pairing of environmental conditioned stimuli with certain pharmacological unconditioned stimuli can result in the elicitation of a response that does not mimic the direct effects of the unconditioned stimulus, but rather appears to be a *compensatory* physiological response. In one test of the conditioning hypothesis, Siegal measured the acquisition of tolerance to morphine (5 mg/kg) using a hot plate test. Three groups of rats received morphine injections under different environmental circumstances during three initial sessions spaced at forty-eight hour intervals. One was injected with morphine and tested on the hot plate when it was in fact hot. A second group received the same treatment except that it was placed on the hot plate surface at room temperature. In a third group the animals received morphine injections but experienced the effects in their home cages and were not exposed to the hot plate. For the critical test, all animals were injected with morphine and placed on the hot plate. The two groups that had had the drug effect paired with the test (i.e., hot plate) cues in the past displayed tolerance in the form of decreased response latencies; in contrast, the animals without such a conditioning history showed no signs of tolerance, *even though they had had the same pharmacological history as the other two groups*. Two other demonstrations enhanced the credibility of this compensatory theory of tolerance. Siegal showed that if a set of environmental stimuli that had previously been paired with morphine injections was later followed by a *saline* injection it was possible to obtain conditioned *hyperalgesia*. This is direct evidence for compensatory response theory, since hyperalgesia is the response that should be compensatory to the direct pharmacologic effect of the drug on the response

to pain. In a final experiment it was shown that tolerance could be reversed by simply exposing rats to environmental stimuli previously paired with morphine but then followed by saline on a series of trials. A control group was provided to show that a comparable period of time of simple withdrawal from morphine without exposure to the drug-associated environmental cues was insufficient to produce loss of tolerance. Presumably, extinction of the compensatory response underlying the development of tolerance was achieved in the experimental group but not in the controls.

These results are intriguing in that they suggest that tolerance can be an exclusive function of environmental control under circumstances in which the pharmacological stimulus *per se* is insufficient to promote an adaptation. However, the ultimate importance of the data is still impossible to assess; a high priority would be to determine whether similar results could be obtained with doses of morphine higher than 5 mg/kg. Clearly, this dose is only a small fraction of that to which rats can ultimately develop tolerance.

#### SUMMARY

This brief survey of the literature on tolerance can be crystallized into three broad summary statements: (a) there are direct analogies between tolerance and other forms of adaptation to stimuli; (b) important aspects of tolerance are subject to alteration by nonpharmacological manipulation, although it does not seem necessary to invoke more than a single underlying mechanism to account for this; and (c) tolerance can be shown to be under the complete control of environmental stimuli in circumstances in which a pharmacological stimulus *per se* fails to provoke tolerance.

At one level, we can summarize these statements by saying that tolerance is a complicated business indeed. But there appears to be something more substantial to be said by way of elaboration. Tolerance seems subject to modification by interventions and processes that must be represented in CNS pathways that are not entirely coincident with the site or sites at which tolerance-producing drugs exert their primary pharmacological effects. It thus seems logical to conclude that there is more to many instances of tolerance than a change in receptor sensitivity at the site of a drug's action. Although the latter may be an important consideration, it cannot be invoked independently of the myriad of CNS events that must be involved in the adaptive interaction of an organism with its environment. The data can lead to no other conclusion.

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## 5. ALCOHOL EFFECTS ON BEHAVIORAL PERFORMANCE

Satanand Sharma, Kenneth Ziedman and Herbert Moskowitz

Southern California Research Institute and University of  
California Los Angeles, Los Angeles, California.

### INTRODUCTION

Alcohol is the most widely used and abused drug in the United States. It is estimated that more than two-thirds of American adults consume alcohol, and thus adult drinking is normal behavior in most circles (1). Such widespread use of alcohol produces a considerable impact on society especially in alcohol related disease-causation, and alcohol effects on functioning as a member of the community. Thus the effects of alcohol on behavioral performance warrants study. A direct concomitant of the drinking behavior is the implication of drinking in the performance of many complex tasks such as driving, flying, and operation of machinery. It is estimated that approximately 50% of the drivers involved in fatal accidents are legally drunk or impaired (2).

### ALCOHOL EFFECTS ON BEHAVIORAL PERFORMANCE

In humans alcohol is rapidly absorbed and distributed to all parts of the body. It exerts its influence on many sites in the body and appears to have multiple mechanisms of action. Therefore, it is difficult to describe the behavioral actions of alcohol in a specific manner based on a single mechanism, and thus few dependable generalizations regarding alcohol action on performance can be advanced. In this review alcohol effects are categorized according to type of tasks.

Since the alcohol literature is voluminous and a great deal of data exists on alcohol effects on performance in humans, no attempt has been made to cite animal data. In the following, human studies have been selected which are representative of alcohol effects on performance. No attempt has been made to present an exhaustive review.

#### Simple Visual Functions

Moskowitz, Sharma and Schapero (3) studied the effects

of 0.69 g alcohol per kg body weight on a variety of visual tests using twelve male subjects. They found that dark adaptation, static acuity, and binocular vision were not affected. In tests of lateral and vertical phoria (position the eyes assume relative to each other and to a fixation target when fusion of the fixation target is rendered impossible), alcohol was found to have no effect on vertical phoria. There was an effect on lateral phoria. The mean prism readings increased by 1.7 diopters. In duction tests, which measure the range that the eyes can turn toward each other, away from each other, and vertically apart from each other in order to maintain single binocular vision of a fixated target, alcohol had no significant effect on supraduction, infraduction and abduction. However, abduction was affected slightly.

Glare recovery, where changes in ambient light levels require the eye to readjust to achieve the previous sensitivity to target contrast at the new level, has been reported to both improve and deteriorate under alcohol (4,5). It is not clear why these contradictory results are obtained but differences in methodology probably are involved. Critical flicker fusion has been reported to show an inconsistent but general trend towards decreased fusion thresholds under alcohol (4). Color preception also changes consistently under alcohol (5). Brightness thresholds have also been reported to be impaired by alcohol (6). Peripheral vision has been reported not to be impaired by alcohol (4,7).

### Auditory System

There is considerable evidence that the auditory system is insensitive to alcohol. Schwab and Ey (8) found auditory acuity not to be affected by alcohol. Signal detectability also is not affected by alcohol (9,10).

### Psychomotor Functions

Several studies have shown that muscular strength is not affected by alcohol (4) but that muscular steadiness is affected at fairly low levels of alcohol (4,11,12). Both auditory and visual reaction times have been found to be consistently affected by blood alcohol levels of 0.1% (11). The Romberg test of body sway has been found to be very sensitive even at low doses of alcohol (4,11). This test requires a person to stand as steadily as possible and body sway is measured. Similarly, hand steadiness is consistently impaired by low doses of alcohol (4). Hand steadiness is most frequently measured as the subject's ability to hold a metal stylus in a small hole in a metal plate without contacting the edges of the plate.

### Division of Attention

Several studies now support the notion that alcohol affects division of attention. Situations which require division of attention are those where input data is received from two or more sources. An example of this is automobile driving where the driver has to concentrate on keeping the car on the road (tracking) and to detect environmental signals such as oncoming traffic.

One study (9) examined attention processes in the auditory modality using 0.69 g alcohol/kg body weight. The experimental tasks included conditions of concentrated attention (subjects attended to input only to one ear) and divided attention (subjects attended to simultaneous input to both ears). Alcohol impaired performance only under the demands for division of attention.

Similar findings have been reported for concentrated and divided attention in the visual modality. Recently, a study by Moskowitz and Sharma (7) examined peripheral vision while the subject was occupied with a central fixation light. There were three central visual conditions: the fixation light was either unblinking, blinked at a slow rate, or blinked at a fast rate. Signal detection was examined at thirty-two points in the horizontal peripheral visual field; at sixteen angles from 12° to 102° on both sides of the fixation light. Alcohol treatments of 0.41 and 0.83 g alcohol/kg body weight were compared with a placebo treatment.

This study specifically tested the hypothesis that the appearance of an alcohol-induced deficit in peripheral vision is a function of the attention or information processing demands placed upon central vision or, for that matter, the demands from any source of information occupying the central processing mechanisms. The condition where the central fixation light was unblinking and thus required no major part of the information processing capacity of the brain duplicates the manner in which earlier studies of peripheral vision under alcohol executed the experiment and failed to find impairment.

The study failed to find any impairment in peripheral vision at either alcohol dose when central vision was occupied with an unblinking fixation light. However, when central vision was occupied with counting the light blinks, there were deficits in peripheral light detections. Under the slow blink central light condition, the two alcohol doses produced 14% and 25% drops in signal detections, and under the fast blink condition 18% and 36% drops in detections. Similar results have been obtained by others (13,14).

### Psychological Refractory Period (PRP)

The PRP has been widely used as an experimental paradigm. It involves the presentation of two stimuli to the subject in close succession with each requiring a response. When the interval between the two stimuli is less than about 300 msec the response to the second stimulus is delayed because it arrives while central capacity is occupied with processing the first stimulus. Thus a delay of the second reaction time reflects an increase in central processing time.

Using this technique a study by Moskowitz and Burns (15) found that 0.69 g alcohol/kg body weight slows information processing.

### Information Processing

Moskowitz and Murray (16) have examined the rate of information processing under alcohol with the visual backward masking procedure. Four letters were presented tachistoscopically for 15 msec duration. After a dark interval (30, 45, 60, 75 msec) a "mask" of letter fragments was presented. This effectively interferes with further processing of the display stimuli from the sensory register into short term memory. When the subject is required to recall the stimulus letters after mask presentation he can recall only those that he had processed from the sensory store into short term memory prior to the onset of the mask. By varying the interval between stimulus and mask and plotting the number of letters recalled against the interval, a processing rate is calculated. Alcohol doses of 0.414 and 0.828 g/kg body weight both slowed the rate.

### Tracking Tasks

Tracking tasks are more typical of perceptual motor performance. Alcohol has been found to affect tracking consistently. Wallgren and Barry (4) found that alcohol produces a decrement in tracking performance and that such a deficit is more likely to appear when the tracking task is performed simultaneously with another task which serves to divide attention.

Sturgis and Mortimer (17) tested subjects on a stylus tracking task and found performance to deteriorate under a blood alcohol level of 0.1%.

Ziedman, Sharma and Moskowitz (18) tested subjects under 0.075% and 0.15% blood alcohol levels with a critical tracking task. This task requires subjects to maintain an illuminated line display on the center of an oscilloscope. This display is made to move in a random manner by a random forcing function. The task becomes more difficult over time so that the subject eventually loses control. This is analagous to balancing a long rod on one's finger. As the rod becomes



shorter the task becomes more difficult. The tracking ability of the subjects deteriorated under both doses.

Aksnes (20) examined performance in a link trainer. Subjects were flying blind and were required to monitor seven instruments as well as a map of the course they were required to maintain. The course imposed limits in regard to altitude, airspeed, vertical speed, turning speed, and time. Subjects were administered either 0.2 or 0.5 g alcohol/kg body weight. The larger dose produced about 0.05% blood alcohol level and appeared to cause an impairment although no statistical analysis was reported.

Hughes and Forney (21) tested performance on a pursuit tracking task with four levels of complexity of the function to be pursued. They administered 0.52 g alcohol/kg body weight resulting in about 0.05% blood alcohol level, and reported that all functions showed large degrees of impairment at this dose level.

Another study (13) combined pursuit tracking with signal detection. They included a condition where additional stress was introduced by noise. Under the quiet condition, the lower alcohol dose of 0.21 g alcohol/kg body weight did not affect tracking scores, but the higher dose of 0.63 g alcohol/kg body weight did impair tracking performance. With the additional stress of noise both alcohol doses produced impairment.

Although most studies of pursuit tracking under alcohol have found impairment, there are a few equivocal studies. In an experiment by Gibbs (22) using a pursuit step-tracking apparatus, which involved steps of unequal probabilities, an alcohol treatment resulting in a peak BAL of 0.10% showed impairment on improbable steps but no impairment on probable steps.

### Eye Movements

One area of ocular motor control which has been universally reported to show the effect of alcohol is the threshold for induction of nystagmus (4). There is a unique form of nystagmus which appears under alcohol which is known as positional alcohol nystagmus or PAN. In this situation an individual tilts his head, (preferably with closed eyes) and a nystagmus develops if the blood alcohol level is in the region of at least 0.06 to 0.08%. The nystagmus involved does not occur without an adequate stimulus; the head must be tilted. In animals and humans who have had bilateral interference with input from the semi-circular canals there is no PAN under the presence of alcohol.

A recent study (23) showed that alcohol at dose levels of 0.5 and 1.0 ml 95% ethanol/kg body weight produced significant reductions in dynamic visual acuity (DVA). DVA is a complex task requiring coordination of sensory and motor

functions in the resolution of detail in moving targets. It has been suggested that the components involved in DVA are static acuity, ocular pursuit of the target by a combination of saccadic and pursuit eye movements (23). It should be noted that static acuity *per se* is not affected by alcohol (2,4).

Another study (24) studied human eye movement electro-oculographically before and after doses of alcohol producing blood alcohol levels of 0.08% and 0.11%. It was found that saccadic and pursuit eye movements where the eyes move rapidly were slowed under both doses of alcohol. The authors reported that the movement of the eyes under alcohol became jerky and inefficient due to replacement of the smooth movement by a succession of irregular saccades. The effect of alcohol on saccadic and pursuit movements raise questions implicating alcohol effects on skills performance which require rapid perception of stationary and moving visual stimuli. One such task is driving.

In a recent experiment (25) the effects of alcohol on visual scanning patterns in a simulated driving situation was determined.

The authors examined eye movements of subjects as they viewed a movie of driving scenes. They used an experimental apparatus with a relatively high data sampling rate (100 per second), a relatively long viewing period (17 + minutes) and a computer data recording and analysis system permitting the rapid extraction of a wide variety of performance variables.

The subjects sat in a driving simulator consisting of the front half of an actual car body facing a 3.66-m wide rear projection screen. Driving films (35 mm) were rear-projected on the screen and subtended a 70 degree horizontal visual angle.

Twenty-one subjects were tested, divided randomly into three groups represented by three alcohol doses, 0.0%, 0.075% and 0.15% blood alcohol levels.

Table 1 shows the allocation of viewing time to dwells, pursuits, saccades, and blinks during the 17 + minutes (1022 seconds) of actual traffic scenes.

For the placebo group, dwells accounted for 64% of total viewing time, pursuits of 19%, and saccades of 14%. In comparison, the alcohol treatment groups showed a trend toward decreased time in dwells and saccades but increase time in pursuits. In contrast to the small changes in total time allocated to dwells, pursuits, and saccades, there were many large changes in the frequencies and mean durations of dwells and pursuits as can be seen in Table 2.

The authors noted that the changes under alcohol can be summarized as (i) an increase in mean time per dwell, (ii) a concomitant decrease in dwell frequency, combined with (iii) an increase in both the frequency of pursuits and mean

TABLE 1

Allocation of Mean Viewing Time in Seconds (Alcohol) <sup>1,4</sup>

<i>Treatment</i>	<i>Dwells</i>	<i>Pursuits</i>	<i>Saccades</i> <sup>2</sup>	<i>Total</i>	<i>Blinks</i> <sup>3</sup>
Placebo	653 (64%)	196 (19%)	140 (14%)	989	33 (3%)
0.075% BAC	601 (59%)	281 (27%)	103 (10%)	985	37 (4%)
0.15% BAC	628 (61%)	259 (25%)	104 (10%)	991	31 (3%)

<sup>1</sup> Total time for traffic portions of movie = 1022 seconds

<sup>2</sup> Total saccadic time estimated by taking the product of total number fixations and mean interdwelt times for each group.

<sup>3</sup> Blinks were counted but their durations were not measured. The time remaining after dwell, pursuit, and saccadic times are summed is attributed to blinks. As a rough indication of the reasonableness of this procedure, note that the mean number of blinks varied from 231 to 316 across groups. If one accepts 0.1-0.2 seconds as the range of blink durations, then an estimate of the range of blink times is 23 seconds to 62 seconds. This range brackets the values of 31 to 37 seconds given in the table.

<sup>4</sup> N = 9 per group

TABLE 2

## Dwell and Pursuit Times (Seconds) and Frequencies (Alcohol)

<i>Measure</i>	<i>Placebo</i>	<i>.075% BAC</i>	<i>15% BAC</i>	<i>Kruskal Wallis Significance Level</i>
Mean time per dwell	0.37	0.47 (+27%)	0.48 (+30%)	.0037
SD	0.046	0.073	0.082	
Mean time per pursuit	1.23	1.48 (+20%)	1.36 (+11%)	.0492
SD	0.17	0.26	0.11	
Dwell frequency	1753	1290 (-26%)	1297 (-26%)	.0042
SD	332	228	122	
Pursuit frequency	157	189 (20%)	192 (+22%)	.0389
SD	75	44	44	
Total time in dwells	653	601 (-8%)	628 (-4%)	NS
SD	104	83	76	
Total time in pursuits	196	281 (+43%)	259 (+32%)	.010
SD	103	98	54	
Mean pursuit length (deg)	5.9	5.5 (-7%)	5.3 (-10%)	NS
SD	0.95	0.49	0.82	
Mean of SD of dwell times	0.32	0.46 (+44%)	0.45 (+41%)	.0110
Mean of SD of pursuit times	0.79	1.06 (+34%)	0.85 (+8%)	.01
Mean of SD of pursuit lengths	3.9	3.9 (0%)	3.2 (-18%)	NS

duration of pursuits. Thus, a person under the influence of alcohol can examine fewer events or examine the same event fewer times. The authors noted that the subjects tend to pursue moving objects more often and for a longer time, further

limiting the opportunity for sharing attention between different events. They conclude by suggesting that the major factor underlying the increased accident potential of alcohol use while driving is the impairment of visual search behavior due to a decreased ability to process information as reflected primarily in increased time necessary for information extraction in dwells and pursuits.

#### Driving Simulation Studies

One very significant aspect of American living is driving. Most American adults drive. Thus the effects of alcohol on driving behavior is important. Studies of driving behavior under alcohol have avoided testing driving-related skills by placing subjects in typical traffic situations for obvious safety reasons. One alternate method of studying driving behavior is by use of driving simulators.

An example of such a study is one by Moskowitz (26). He placed subjects in a car where they were required to view a large circular screen depicting a traffic movie. The subject's manipulation of the accelerator controlled the speed of the movie projection, and his manipulation of the steering wheel moved the projected image laterally. Braking slowed the speed of the movie. The subject "felt" as if he was in a real driving situation. Twenty-five performance measures of car control and tracking were derived (such as steering movement, brake actuation and speed). None of the parameters showed any impairment under 0.1% blood alcohol level. The study was then replicated with the inclusion of a simple subsidiary task which required the driver to respond appropriately to two colored lights presented at one of two positions on a random basis, with a frequency of one per minute during a thirty-one mile drive. Under the additional requirement for information processing of the subsidiary task, alcohol produced a decrement in signal detectability of the subsidiary task as well as impairment of twelve of the twenty-five car control measures. The author suggested that the alcohol effect was due to alcohol induced impairment of the capacity for information processing, especially as required in time sharing of several concurrent tasks.

These results are in agreement with those of another study (27) where alcohol effects were assessed in an actual flying situation.

In this study by Billings et al. (27), sixteen subjects took off, instrument-flew and landed a plane under four alcohol treatments, resulting in 0, 0.04, 0.08 and 0.12% blood alcohol levels. Eight of the subjects were highly experienced professional pilots, while the other eight were fairly experienced non-professionals. Flights took place with

a safety co-pilot plus a physician located behind the pilot in order to incapacitate him, if necessary. Although the tracking demands of flying are more difficult than those of driving, the experienced pilots suffered no significant decrement in their tracking ability even at the highest dosage. However, beginning at the lowest dosages they committed procedural errors which were a hazard to flight. At the highest dose level, the safety co-pilot had to take command of the plane eleven times to prevent an imminent accident. The inexperienced pilots exhibited impairment in their tracking skills and accumulated far more procedural errors including taking off with full flaps, flying without lights, taking off with carburetor heat on, turning the wrong way in response to instructions, and flying a landing approach tuned to the wrong frequency. Catastrophic procedural errors included loss of control in flight, turns toward oncoming traffic and landing errors that would involve striking the ground.

The authors noted the pilots placed tracking or guiding the aircraft as their primary task and relegated all other operations to secondary tasks. They pointed out that as the pilots were progressively affected by alcohol, they became progressively less able to cope with the various facets of their task, and the secondary tasks were the ones that suffered first and the most.

It is clear from the foregoing that alcohol affects a wide variety of performance tasks, from simple reaction time to complex operations such as flying an aircraft. It is noteworthy that alcohol is widely distributed throughout the body, to all tissues and fluids of the body. Alcohol is present in the plasma, in erythrocytes and in the cerebrospinal fluid. It primarily affects the central nervous system. Thus, alcohol is available at most cells of the body and while the exact nature of its interaction with the cells is still unknown, it influences a wide and disparate range of physiological and behavioral mechanisms.

In summary, it is clear that alcohol impairs performance, especially that requiring division of attention. Psychomotor tasks are only slightly affected (28). The ocular motor effects, the slowing of information processing rate, and the deleterious effects of alcohol on other preceptual tasks make it hazardous to perform complex tasks under the influence of alcohol.

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## 6. TERATOGENIC EFFECTS OF *IN UTERO* ETHANOL EXPOSURE

Carrie L. Randall

Medical University of South Carolina, Department of Psychiatry  
& Behavioral Sciences, Charleston, South Carolina, 29401.

### INTRODUCTION

In its broadest sense, teratology can be viewed as the study of monstrosities, and a teratogen as an agent that causes abnormal development. Interest in malformations in both man and animals has been evident since the earliest days of recorded history, but detailed scientific studies of the phenomenon, especially with regard to ethanol, were not introduced until the early 1900's. These pioneer investigators were not interested in ethanol or alcoholism, *per se*, but rather were embryologists or environmental teratologists concerned with the study of developmental abnormalities. In an attempt to identify possible teratogens, a number of agents were used. As might be expected, ethanol was among them.

Initially, non-mammalian species were commonly employed subjects. Since fertilization and subsequent development occurs externally in such species, rather than internally as in mammals, experimental observation and intervention was convenient. Embryos bathed in ethanol solutions demonstrated abnormal cleavage, stunted growth, and central nervous system disturbances, often following atypical and predictable pattern (1). However, effective concentrations were usually five to forty times higher than the highest levels reported in man and are, therefore, of questionable significance.

### PLACENTAL TRANSFER

Since alcohol is a small, readily diffusable uncharged molecule that is equally distributed in the body water, it is not surprising that it easily crosses the placenta and reaches the fetus. Unlike many other drugs, alcohol distribution is not influenced by degree of lipid solubility. Nicloux (2,3) was the first investigator to demonstrate that alcohol ingested by a gravid woman, dog, or guinea pig passed to the fetus in concentrations paralleling those circulating in maternal blood.



These pioneer studies by Nicloux have been confirmed and extended using a higher degree of sophistication than was available at the time. Gas chromatographic analysis demonstrated a rapid equilibration between the mother and fetus in the pregnant ewe (4), while similar findings have been reported using autoradiographic techniques in humans, as well as monkeys, hamsters, and mice (5,6,7). Autoradiographic examination further revealed a pattern of distribution involving most organs, with the highest concentration in the liver, myocardium and bone marrow (7).

It is evident from these studies that the placenta, even late in pregnancy, does not constitute a barrier to ethanol as it does to many other drugs. On the other hand, acetaldehyde, ethanol's major metabolite, does not cross the placenta in appreciable amounts, at least in the near-term rat (8). Rather, it has been suggested that the placenta acts as a metabolic barrier by metabolizing significant amounts of acetaldehyde (9), which may be potentially toxic to the developing fetus.

#### FETAL AND NEWBORN METABOLIC CAPACITY

Since the placenta does not block ethanol penetration to the fetus, it is concluded that the fetus is exposed to blood alcohol levels similar to maternal levels. If results from the rat can be generalized to the human, the *in vivo* rate of ethanol metabolism is not affected by pregnancy (10).

Prompted by the popular clinical use of ethanol to prevent premature labor through inhibition of oxytocin, Seppala (11) and Idanpann-Heikkela (12) studied the elimination of ethanol in the pregnant female, fetus and newborn. A gas liquid chromatographic method of analysis indicated the equilibrium between maternal and fetal concentrations was reached sixty minutes after the initial infusion.

At birth and up until thirty minutes post-delivery, the blood alcohol levels in the mother and fetus remained elevated; by four hours post-partum the maternal elimination rate was twice as high as the newborn's. Premature infants infused with 8% w/v ethanol for up to forty minutes post-partum eliminated alcohol in a linear function (13). Higher, but decreasing blood alcohol levels in the fetus and newborn suggest that the near-term fetus and newborn are capable of metabolizing ethanol in the typical zero-order function, but not at adult capacities.

Although the activity of liver alcohol metabolizing enzymes is not necessarily a good predictor, or sufficient explanation, of metabolic capability for ethanol elimination, interesting reports are worthy of mention. In the rat, alcohol dehydrogenase (ADH) activity was minimally

detectable at eighteen days gestation and reached adult levels by eighteen days of age (14). In aborted human fetuses, 3-4% of the adult liver ADH activity was evident in eight week old livers and adult levels were reached by five years of age (15). Structural differences between adult and fetal liver ADH seem to exist (16-18). The few studies concerned with liver aldehyde dehydrogenase activity report considerably lower enzyme activity in the human and rat fetal liver than the adult (9, 14). Interestingly, higher circulating levels of acetaldehyde have been reported in peripheral blood from pregnant rats than non-pregnant rats (19), but no detectable acetaldehyde was measured in near-term fetuses (8) whose mothers were given alcohol acutely by injection.

It can be suggested from the literature cited that the near-term fetus and newborn are capable of metabolizing ethanol and acetaldehyde, but at a significantly slower rate than the adult. Younger fetuses, however, do not have the capacity to metabolize drugs and are dependent upon the mother for drug elimination. The significance of exposure to high circulating alcohol and acetaldehyde levels *in utero* on subsequent development is not understood, but deleterious effects on both physical and mental status, as well as more subtle biochemical effects are clearly suggested. The remainder of this review will cite evidence to support the notion that alcohol is a teratogenic agent.

#### STILLBIRTHS/RESORPTIONS

A dramatic, but often overlooked, measure of teratogenesis is prenatal/postnatal mortality. A high incidence of miscarriages and stillbirths has been ascribed to alcoholic parents (20-23). Two separate issues arise, depending upon which parent was the alcoholic. Often, however, only one parent is considered while drinking habits in the other are ignored (23).

Miscarriages can thus arise from inherited, prenatal, or environmental factors, or any such combination. Until these variables can be partitioned adequately, alcohol, *per se*, cannot be implicated as responsible for miscarriages or stillbirths. A careful statistical evaluation of the incidence of miscarriages and stillbirths in the general population, as well as in the offspring of non-alcoholic women matched for age, parity, physical condition, and socio-economic standing, is needed before any definitive conclusions can be drawn. However, a potential problem in gathering accurate statistics on this issue in alcoholic women is the absence of perinatal care in the lower socio-economic groups. Miscarriages may often go undetected.

This issue could be more readily resolved with adequate

animal models, for species such as the rodent tend to resorb, rather than to abort, fetuses, and implantation sites can readily be quantified and compared to controls. Unfortunately, few experimenters have chosen this approach to the issue. A plethora of reports describe an increased number of prenatal deaths, stillbirths, and decreased litter size and weight calculated after birth, in fetuses exposed prenatally to ethanol (24-28).

Rodents often cannibalize their young, especially if visceral organs are exposed. Parity as well as environmental conditions also influence pup-killing (29). It is, therefore, possible that defective pups were killed before counted. Further, early resorptions are not reflected in post-partum evaluation. Even palpations and subsequent verification at birth (30) can be complicated by repeated handling of the mother and cannot accurately be detected during early stages of gestation.

Another key issue is the fact that alcoholics tend to be undernourished and protein deficient, since ethanol serves as a caloric source. Nutritional variables, then, complicate the picture. Few studies, either in humans or animals have attempted to control for this crucial variable.

The liquid diet technique originally proposed by Freund (31) circumvents this criticism by allowing for a pair-fed control group that receives the same total number of calories per day as the ethanol group, with ethanol-derived calories replaced by sucrose. Since all other variables (e.g. handling and stress) are assumed to be similar for the two groups, differences between them can be attributed to the presence of ethanol. Chernoff (32) utilized the liquid diet procedure of alcohol introduction in CBA and C3H mice and reported dose-related strain differences in ethanol's embryotoxic effect. Although not specified, it is assumed that he utilized the pair-feeding paradigm in his controls.

Our own work with this technique has demonstrated an increased number of resorptions in ethanol-treated primiparous C57BL mice, as compared to lab chow and sucrose pair-fed controls (33). The total number of resorptions increased proportionally to the increased amount of ethanol ingestion; with diets containing 35% of the total calories as ethanol, few viable litters were recovered (34).

From these recent animal models controlling for nutritional variables, ethanol's embryotoxic potential is clearly implicated. It is possible that different periods of exposure, dose, and parity, as well as species or strain differences, may influence the number of resorptions. The significance of these reports remain, however. In the presence of controlled nutrition, ethanol-treated females resorb significantly more fetuses than pair-fed sucrose or lab chow controls.

### PHYSICAL/MENTAL STATUS

The most blatant expression of embryopathic drug action is abnormal physical or mental development of the progeny. Since the time of the Greeks and Romans, parental ethanol abuse at the time of conception has been held responsible for skeletal and mental defects in children. Hence, ethanol was prohibited on the wedding night (35).

This point of view was pervasive in the literature, with reports constantly emphasizing examples of such defective children and implying an overall degeneration of the human race (36). The basic premise was that alcohol acted in some deleterious way to alter the egg or sperm. Chromosomal aberrations, or basic inheritance of birth defects is a separate issue, not necessarily similar to *in utero* exposure to ethanol. Further, even if the fetus were exposed *in utero*, from a perusal of these studies, two things become obvious. First, the reports are generally clinical observations. Accurate history of pattern of maternal drinking habits, socio-economic status, dietary regimen, perinatal care, marital status, drug use, or parity are frequently not included in the records. Second, the paternal history is omitted. It thus becomes impossible to assess the relative overall contribution of ethanol, *per se*, to the resultant defective children. Animal models should have afforded better control of these variables, but unfortunately were plagued by other confounding variables.

Combemale (37) published one of the earliest reports of *in utero* exposure to alcohol in mammals. Two normal dogs were mated; the bitch was exposed to ethanol for the first twenty-three days of gestation. Three of the six pups were stillborn; the remaining three were of "weak intelligence" and when mated to "normal intelligence" studs, produced defective young. This early report was followed by others suggesting deleterious effects of ethanol on immature organisms (38-40).

Stockard, however, reported the most extensive research in this area (41-45). He devised a specific apparatus for administering alcohol to pregnant guinea pigs without handling them. Briefly, the animal was prodded into a chamber filled with vapors from 95% ethanol and left until "intoxicated" for six days/week. "Defective" young were often found, affected particularly in the hind extremities. Eye defects were also very common, such as opaque cornea, opaque lens, and anophthalmia.

Pictet (46) and Durham and Woods (47) criticized Stockard's work on a number of grounds and set out to re-examine the issue. They argued that similar defects are characteristic of certain lines of guinea pigs and cannot validly be attributed to maternal ethanol treatment. Their different

initial stock, method of intense inbreeding and lack of age and parity controls make these findings also of little significance.

This issue continued to foster empirical studies, extended to the mouse (48), chicken (49) and rat (50,51). Similar methods of intoxication were utilized (*i.e.* inhalation), and when inconsistent results were obtained, differences were attributed to species variation in sensitivity to ethanol.

Interest eventually waned in this area, especially when reports demonstrating "superior" offspring from ethanol-treated parents appeared. Ethanol was proposed as a selective agent allowing only the fittest of the species to survive (52,53).

Only recently has general interest been renewed in the distinct possibility that ethanol itself may result in abnormal physical and/or mental development of the young. LeMoine and his colleagues in France, described a common syndrome in children of alcoholic mothers (54). Of 127 children born to alcoholic parents twenty-five were malformed; growth deficiencies were evident from birth and psychomotor retardation was apparent. I.Q. scores averaged about seventy; EEG recordings were atypical.

Ulleland was one of the first to describe a similar clinical observation in children of alcoholic females in the United States (55). She observed six small-for-gestational age infants, all of whom had alcoholic mothers. Prompted by this coincidence, Ulleland examined the records of deliveries at Harborview Medical Center (Washington) for an eighteen month period. Forty-seven underweight children were born to a total of 1,582 mothers; ten of the twelve mothers were over thirty-five. Since this hospital services poor or underprivileged clientele, inadequate nutrition could have been responsible for the low birth weight in the offspring as well as maternal medical problems, drug abuse, or environmental variables. Eight of eleven mothers when examined post-partum had mild-severe protein or caloric deficiency, presumably also present during pregnancy.

Jones *et al.* (56) extended these observations. Initially eight unrelated children born to alcoholic mothers were observed. Six of the eight children were ascertained because of similar physical abnormalities and the remaining two subjects because their mother was an alcoholic; three children were American Indians, three Negroes, and two Caucasians, ranging in ages from eleven weeks to four years. The duration of alcoholism in the mothers was two to twenty-three years, with and average of 9.4 years.

All patients demonstrated impaired pre- and post-natal growth. Common craniofacial and limb defects were observed, such as short palpebral fissures, protrusion of one or both jaws, epicanthal folds, limitation of joint movement, altered

palmar crease patterns, and cardiac abnormalities. Assessment of mental, motor and social development revealed low performance, better correlated with mental age than chronological age.

A subsequent report (57) identified three more children with similar malformations, all of American Indian descent. Some new abnormalities reported were cleft palate, respiratory difficulty, hypoglycemia, hypocalcemia, and hyperbilirubinemia. The death of one subject allowed a necropsy. The brain was incompletely developed, the cerebral cortex in particular, and lacked a corpus callosum. Neuronal migration was reported to be disoriented. Subsequent reports by others corroborated these initial findings (58-61). This pattern of altered growth and morphogenesis is now commonly referred to as the "fetal alcohol syndrome".

A follow-up study of twenty-three offspring of alcoholic mothers (62) resulted in some profound implications. The subjects were drawn from a population of 55,000 women studied by the National Institute of Neurologic Disease and Stroke. The charts of the mother and child were examined with no direct patient interview. The population represented eleven negroes, eleven caucasians, and one American Indian, ranging in age from twenty-one to forty, and of lower socio-economic class.

Seventeen percent of the twenty-three offspring included in the study died before one week of age. Of the remaining nineteen subjects, only thirteen were available for evaluation at seven years of age. Intellectual impairment was the most common occurrence with 12% of the cases three standard deviations below the mean and 63% between one and two standard deviations below the mean. Half of the subjects remained with their mother while the other half lived with relatives or in a foster home. Those remaining with their biological mother tended to have lower I.Q. scores. Six subjects were diagnosed on the basis of physical abnormalities to have the "fetal alcohol syndrome". The authors suggested that since 43% of the subjects were adversely affected (four dead and six with the fetal alcohol syndrome) the consideration should be given to termination of pregnancy in chronic alcoholic women. Some investigators have questioned the validity of this suggestion (63). A recent up-date now includes a total of sixty cases of the fetal alcohol syndrome (64).

These sensational reports of a common syndrome of dysmorphology in children of alcoholic women suggest the distinct possibility that ethanol is teratogenic to the human fetus. As cautioned by Green (65), additional variables may complicate the picture. Economic status, nutritional status, drug use parity, and prenatal care are extremely difficult to control in a clinical situation. Thus, it becomes

inherently difficult to ascertain the relative contribution of ethanol, *per se*, to the observed deleterious effects.

The animal experiments initially designed to assess the effect of maternal alcohol consumption on fetal development are, unfortunately, plagued by a common criticism of the clinical reports, that is, no nutritional controls (66-71), and will not be reviewed in detail. It can generally be concluded from these reports that maternal ethanol consumption results in decreased birth weight and/or number, retarded development, and impaired learning ability. Data from the open-field apparatus yields conflicting results (69,72).

More recently, Chernoff (32) reported a mouse model of the fetal alcohol syndrome attempting to circumvent the nutritional issue by feeding ethanol to mice in a nutritionally balanced liquid diet representing from 0-35% of the total calories as ethanol for at least thirty days prior to and throughout gestation. Strain differences existed. Alcohol-treated CBA progeny demonstrated open eyes and deficient ossification at a concentration supplying 25% of the total calories as ethanol; there was an increasing incidence of cardiac and neural anomalies with higher concentrations evident in both CBA and C3H mice. The author implicates strain differences in alcohol dehydrogenase activity as a possible explanation for the observed teratogenic and embryotoxic effect, although metabolic rates and/or acetaldehyde levels were not reported.

Kronick (73) also administered ethanol to pregnant mice and observed a high incidence of anomalous development in the progeny. B6D2F<sub>1</sub>/J mice were injected intraperitoneally with approximately 5.7<sup>1</sup> g/kg ethanol on gestation days 8, 9, 10 and 11, or singly on one of the gestation days 7 through 12. External malformations were evident in 27% of the females treated on days 8 and 9 and 57% of the females treated on days 10 and 11. Single injections on day 9 and 10 produced the highest incidence of abnormal fetuses (41/68 and 26/51 respectively). Anomalies included coloboma of the iris, extrodactyly of the forepaws, hydronephrosis, hypoplastic atria, and exencephaly.

Our own work is also concerned with an animal model approximating the fetal alcohol syndrome in mice (C57BL/J6). We hypothesized that if alcohol acted similarly to other teratogens (74), its effects should be demonstrable during a finite period of critical development. For this reason we chose to introduce the ethanol on gestation day 5 (immediately following implantation) and remove it on gestation-day 11. Our method of drug exposure was via a nutritionally balanced liquid diet containing either 17, 25 or 30% of the total calories as ethanol. We pair-fed controls a similar diet isocalorically substituted with sucrose instead of ethanol.

Fetuses were removed on gestation day 19 by caesarian section and examined for external and internal anomalies (75). We found a dose-related increase in anomalous fetuses as the proportion of ethanol-derived calories increased to 30%. Nearly total resorptions were evident at higher concentrations.

The pattern of anomalies was similar across doses, but the incidence varied. We observed frequent limb anomalies, including adactyly, syndactyly, and ectrodactyly of the forelimbs, cardiovascular anomalies including abnormalities of both the major branches of the aorta and vena caval system and intercardiac anomalies, such as atresia of the mitral valve and interventricular septal defects. Hydronephrosis and hydroureter of varying degrees was also commonly observed, as were microphthalmia. Other anomalies included gastroschisis, exencephaly, hydrocephalus, and anophthalmia (34).

Our results, and those of Chernoff (32) and Kronick (73) clearly demonstrate the teratogenicity and embryotoxicity of ethanol in the mouse. Our results in particular further demonstrate this effect in the presence of adequate nutrition, a common criticism of the clinical reports. The anomalies observed by us and others in animal models are strikingly similar to those reported in children of alcoholic mothers (76).

#### MISCELLANEOUS EFFECTS

There is no reason to believe that all teratological effects should be obvious at birth. In addition to gross structural and physical anomalies, more subtle alterations in body chemistry and function probably exist, but either are latent or not manifest until challenged and, therefore, are not immediately recognizable post-delivery. Since the identification and characterization of the fetal alcohol syndrome is relatively recent, a paucity of data exist on possible latent effects (62). In fact, many of the children identified to date have not yet reached puberty (76).

Animal models have yielded little information on long-term pathogenesis or functional anomalies except for reproductive capacity (30). Changes in behavioral variables (69, 72,77), EEG (78,79), neurochemistry (80,81), acid-base balance (82), seizure susceptibility (72), liver enzyme activity (83) and alcohol preference (69) have all been demonstrable shortly following birth or weaning. Whether these effects are permanent, or reversible with age is unknown.

#### POSSIBLE MECHANISMS OF ACTION

It is strongly suggested, from both clinical and empirical reports, that ethanol is a teratogenic agent capable of



producing a variety of developmental anomalies. Since the first report by Jones et al. (56) describing the fetal alcohol syndrome, an additional fifty-two children have been identified as having the syndrome. Clinicians have been amply warned not to confuse the fetal alcohol syndrome with other similar syndromes (84). The fetal alcohol syndrome has been adequately identified, characterized, and publicized to both the professional and layman, and is finally receiving some credence. The important issue now is to determine its etiology.

Ethanol, *per se*, is most often implicated as the toxic agent in producing the anomalies (64,84). As indicated earlier, it rapidly crosses the placenta and reaches the fetus in amounts approximating those in maternal blood. It is, therefore, a likely prospect, although a variety of alternative possibilities exist.

The role of malnutrition-induced anomalies is casually refuted, although alcoholic women are often either malnourished or undernourished, lacking adequate vitamins, minerals and protein (56). The argument is that the pattern of sustained growth deficiency is not similar to that reported in children of malnourished mothers. Recent animal experiments performed under controlled nutritional conditions have more convincingly ruled out nutritional factors as the primary teratogen by demonstrating embryopathology and toxicity in the presence of adequate diet, but the possibility still remains (32,34,73).

Social and environmental factors may also be responsible for the anomalies observed in children of alcoholic mothers. For example, strenuous work during gestation or accidental falls may result in defective children as well as premature births. Premature birth may, in fact, be responsible for some of the deficiencies observed. Proper prenatal care is usually minimal in the majority of women alcoholics and since the fetus is abnormally small, the exact duration of gestation may not be accurately determined.

Maternal age and parity may also be significant variables. Many of the mothers reported on were over thirty-five years old (64). Multiple drug use must also be considered as another viable alternative explanation for the anomalies observed.

The importance given acetaldehyde, ethanol's major metabolite, is minimal, even though acetaldehyde in high doses is toxic and may be responsible for effects previously attributed to ethanol (85). Kesaniemi and Sippel (8) recently reported a placental barrier to acetaldehyde in the rat. These authors also reported significantly higher circulating levels of acetaldehyde in pregnant rats as opposed to non-pregnant rats (19). They propose that the placenta metabolizes acetaldehyde before it reaches the fetus and protects it from possible toxicity. These experiments were performed in the

near-term fetus, however.

In the rodent the placenta continually changes with increasing gestation. What may hold true for the last trimester when the chorioallantoic placenta is the functioning placenta may not necessarily be true for earlier stages of gestation characterized primarily by the yolk-sac placenta. In light of the recent results from animal models, teratogenic effects of ethanol are evident with second trimester exposure alone (34, 71,73). Determination of acetaldehyde levels in fetuses at this time period is critical, before acetaldehyde can validly be rejected as the teratogenic agent. Direct or indirect toxic effects produced by acetaldehyde remains a viable alternative for the anomalies observed in the fetal alcohol syndrome.

The teratogenic effects of a drug need not be a result of direct embryotoxicity; indirect effects can be equally deleterious. Alterations in the normal maternal-fetal interaction may be critical to normal development.

Ethanol may render the pregnant mother protein and/or vitamin deficient. Folic acid levels may be low. Acid-base balance may be impaired. Blood pressure, and therefore oxygen transfer, may be expected to change with high doses. Any of these variables can result in anomalous development (86-89).

Probably the most important indirect drug effect is the effect on the transport mechanisms of the placenta. Placental dysfunction or morphological changes most probably result from chronic alcohol abuse and high acetaldehyde levels, although this issue has yet to be examined.

The mechanism of action, critical period, and toxic dose level responsible for abnormal development in children of alcoholic women are all within the realm of scientific experimentation. Unlike other teratogens (e.g. trypan blue) the basic actions of alcohol and to a lesser degree, acetaldehyde, are documented (90). Prospective studies in the clinical setting, with adequate control groups, can offer answers to basic questions from verbal self-report data regarding history, duration, and pattern of drug use as well as environmental factors that animal models will not adequately simulate. Animal models, on the other hand, should soon offer insight into the possible biochemical and physiological etiology of ethanol-induced changes in organogenesis. The ultimate goal of identification is subsequent intervention and prevention of ethanol's deleterious effect on the developing fetus.

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## 7. PHARMACOGENETICS OF ALCOHOLISM

Richard A. Deitrich and Allan C. Collins

Department of Pharmacology, School of Medicine, University of Colorado, Denver, Colorado, 80220 and School of Pharmacy and Institute for Behavioral Genetics, University of Colorado, Boulder, Colorado, 80309.

### INTRODUCTION

It has long been recognized among the laity that vast differences in response to ethanol exist among humans. For many years, different individual responses to ethanol were considered to be accidental, their occurrence in groups was not reported, and there was no suggestion that a rational explanation for individualized response might be possible. New light was thrown on the multiplicity and diversity of responses to drugs in general when clinical and research attention was directed toward the incidence of individual differences in response to drugs within families and other groups. Numerous investigations have clearly established a genetic influence on variability in drug response. The field of study which deals with this phenomenon is called pharmacogenetics. Evidence is now available to demonstrate that an individual's response to alcohol, like that to other drugs, is influenced by genetic factors. In this chapter, we shall present selected studies from the literature and our own findings with respect to the pharmacogenetics of alcohol related behaviors in humans and other animals. We shall also suggest how genetically influenced differences in these behaviors may be used as a tool to aid in the elucidation of the underlying mechanisms by which alcohol exerts its actions. In general, those studies which provide biochemical explanations will be emphasized.

### DEFINITIONS

The following definitions are provided for those readers who may not be acquainted with the genetic and behavioral terms or the biochemical pathways under consideration. They are intended specifically for this presentation, and more technical definitions may be found in appropriate texts.

### Alcohol Acceptance

For this method of assessing alcohol consumption, the usual procedure is to deprive animals of drinking fluid for 24 hours and then present them with a specified ethanol solution. The amount consumed in a certain period of time is compared to predeprivation water consumption to provide a measure of acceptance.

### Alcohol Preference

There are numerous variations of this basic procedure for assessing alcohol consumption. Usually, a two-bottle choice situation is used--one bottle contains a specified ethanol solution, and the other contains either water or a particular control solution. The positions of the bottles are changed daily according to a prearranged schedule. The amount consumed from each bottle is recorded each day to obtain a measure of alcohol preference. Preference may be expressed in terms of volume of ethanol solution consumed, volume consumed relative to body weight, or ratio of volume consumed to total liquid consumption.

### Allele

One of two or more alternate forms of a gene occupying a locus.

### Biochemical Pathways

Ethanol Metabolism:  $\text{CH}_3\text{CH}_2\text{OH} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{CH}_3\text{CHO} + \text{NADH} + \text{H}^+$

Acetaldehyde Metabolism:  $\text{CH}_3\text{CHO} + \text{NAD}^+ \xrightarrow{\text{ALDH}} \text{CH}_3\text{COOH} + \text{NADH} + \text{H}^+$

Amine Metabolism:  $\text{R-CH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{MAO}} \text{R-CHO} + \text{H}_2\text{O}_2 + \text{NH}_3$

### Enzymes

*Alcohol Dehydrogenase (ADH)*. A group of isozymes occurring primarily in the cytosol of the liver and responsible for most of the metabolism of ethanol. Pyrazole is an inhibitor of these enzymes.

*Aldehyde Dehydrogenase (ALDH)*. A group of isozymes occurring primarily in the liver but also in other tissues. There are at least three isozymes in the cytosol of the liver. One of these enzymes can be induced in mice and in some lines of rats by phenobarbital and DDT. Another liver cytosolic enzyme can be induced in rats by tetrachlorodibenzo-p-dioxin

(TCDD). Liver mitochondria contain at least two isozymes. There is a matrix enzyme which has a high affinity for acetaldehyde (low  $K_m$ ) and is primarily responsible for acetaldehyde metabolism. This enzyme is inhibited by pargyline and by some other monoamine oxidase inhibitors. Liver mitochondria also contain an enzyme between the inner and outer membranes that has a high  $K_m$  for acetaldehyde. In addition, liver microsomes contain an aldehyde dehydrogenase. All of these enzymes are inhibited by disulfiram (Antabuse).

*Monoamine Oxidase (MAO).* An enzyme located on the outer mitochondrial membrane that is responsible for oxidizing many biogenic amines (such as epinephrine, norepinephrine, dopamine and serotonin). It is inhibited by pargyline, nialamide and several other compounds.

### F1 Generation

The offspring of a cross between two inbred parental strains. These animals are heterozygous at each locus at which the parental strains differed. They are all genetically uniform.

### F2 Generation

Progeny produced by the mating of two individuals of an F1 generation. Alleles of genes that are heterozygous in the F1 generation can segregate in the F2, and each individual F2 animal is a genetically unique event. However, genetic variability is a function of the particular parental strains that produced the F1 generation.

### Genotype

The genetic constitution of an organism, as distinguished from its observable characteristics (phenotype).

### Heritability

A measure of the proportion of phenotypic variance that is due to genetic variability among individuals in a population. The measure is descriptive of a particular population in a particular environmental milieu.

### Heterogeneous Stock

Genetically heterogeneous populations can be established in various ways. A commonly used method is to intermate a number of inbred strains, with subsequent maintenance of the

stock through random mating or some other outbreeding scheme. A heterogenous stock is useful for normative studies; also, such stock is often used as the foundation population for a selective breeding program.

### Heterozygous

Having two different alleles at a locus.

### Homozygous

Having identical alleles at a locus.

### Inbred Strain

Offspring of individuals that are more closely related than would occur if mating were at random in a population; usually, inbreeding denotes the mating of siblings, and a strain that has been propagated by brother-sister mating over 20 consecutive generations is commonly designated as inbred. All individuals are descendants of one female and her brother. They are homozygous at all loci. The result of such inbreeding is a reduction of genetic variability to the point where, for practical purposes, each individual can be regarded as genetically identical to each other individual. It is important to note that the process of inbreeding is nondirectional; that is, the particular genetic configuration that is fixed in a strain is a matter of chance, except for characters related to reproductive fitness. See McClearn (1) for a discussion of inbreeding and other mating procedures used in alcohol research.

### Locus

Position on a chromosome occupied by allelic forms of a particular gene.

### Phenotype

The observable properties of an organism. These properties are produced by the genotype in conjunction with the environment.

### Selective Breeding

A mating procedure which may produce populations that differ in a particular phenotypic attribute. In other words, it is a directional process. All animals in a genetically heterogeneous population are measured for the trait in

question; females displaying high levels of the trait are mated with males showing high levels, and females displaying low levels are mated with males showing low levels. Thus, two lines are begun, and they are maintained by mating the highest males and females of the high line and the lowest of the low line in subsequent generations. To the extent that genetic factors influence the variability of the trait in the foundation population, the lines become increasingly divergent with respect to the trait in succeeding generations. Continued selection may lead to increasing homozygosity within lines for the locus influencing the phenotype; however, if heterozygosity at a locus which influences the phenotype contributes to a high or low level of the trait, homogeneity will not be achieved within lines.

#### *ANIMAL MODELS OF ALCOHOL-RELATED BEHAVIORS*

Genetic influences upon several alcohol-related behaviors are especially evident. Specifically, preference for alcohol, initial (first dose) sensitivity to alcohol, and the development of physical dependence upon alcohol all appear to be influenced by genetic factors. Direct evidence to support the contention that heredity influences these behaviors has been obtained from both animal and human studies. The use of animal models has been criticized on the grounds that they do not completely mimic the human condition. Nevertheless, there is no doubt that animal models of various aspects of human alcoholism do provide much important information.

##### Preference for Alcohol

Studies concerned with alcohol preference in humans are relatively few [see Varela (2) for a discussion of human appetites for alcohol]. However, preference for alcohol is the most intensively studied of the genetically influenced alcohol-related behaviors in laboratory rodents. Two approaches, typifying much of the work in pharmacogenetics, have been used to demonstrate a genetic influence on alcohol preference. In the first approach, the investigator takes advantage of existing laboratory strains (or of different species that exist in nature). The second approach is to apply the selective breeding procedure to a particular behavioral or biochemical trait of interest. The latter method (see definitions) is much more tedious and time consuming. When properly carried out, however, it provides an extremely useful tool for the study of behavioral or biochemical traits.

McClearn and Rodgers (3) utilized the first of these approaches to assess the alcohol preference of several inbred

mouse strains, whereas Eriksson (4,5) applied the second approach and bred rats selectively for alcohol preference. These investigators, the former in the United States and the latter in Finland, assessed alcohol preference in a similar fashion (see definitions). McClearn and Rodgers observed a substantial difference in preference for 10% alcohol solution among various inbred mouse strains. The C57BL/Crg1 strain showed significant preference for alcohol, while A/Cal, BALB/c and DBA/2 animals preferred water and avoided alcohol. Intermediate preference was shown by the C3H/HeCrg1 strain.

Major advantages of using inbred strains of mice or rats are their general availability and their constancy over time and in different laboratories. For example, many studies have replicated some of the strain differences in alcohol preference originally noted by McClearn and Rodgers. These include investigations by Rodgers et al. (6), Fuller (7), McClearn and Rodgers (8), Eriksson and Pikkarainen (9) and Pickett and Collins (10). The disadvantage of the use of inbred strains is that the process of inbreeding is non-directional (see definitions). The strains will differ with respect to many characters, some of which may be related to the preference difference and some of which may not. As a result, relationships between preference and any other behavioral, biochemical or pharmacological trait are likely to be fortuitous. Only by other means of genetic analysis can such correlations be tested.

Further evidence that alcohol preference is genetically influenced comes from studies in which inbred strains were crossed to produce filial generations. McClearn and Rodgers (8) obtained an F1 generation from a C57BL x DBA cross and found that these animals exhibited intermediate preference. Thomas (11) observed that the preference of animals in the F1 generation produced by a C57BL x DBA cross was more similar to that of the parental DBA strain. A subsequent F2 generation showed a wide range of alcohol preference, with most of the animals exhibiting high preference. However, when Pickett and Collins (10) developed an F2 generation from a C57BL x DBA cross, they obtained a majority of low-preferring animals. In addition to providing further evidence of a genetic influence on alcohol preference, these studies also suggest that a number of genes, perhaps working in an additive fashion, are involved in this behavior. Whitney et al. (12) reanalyzed much of the early preference data and concluded that early estimates of heritability were erroneously high. These authors suggest that a value for heritability that ranges between 0.10 and 0.15 may be a more realistic estimate.

As previously noted, Eriksson (4) developed two selectively bred lines of rats which differ in alcohol consumption.

The high-preferring line is referred to as AA (alcohol addicted), and the low-preferring line is designated ANA (alcohol non-addicted). It should be noted that the original designation "alcohol addicted" is inappropriate according to more recent usage of the term "addicted", since it implies that these animals are inherently addicted, which is clearly not the case, or that they are easily addicted upon exposure to alcohol, which also has not been demonstrated. Still, because the designations AA and ANA are used by Finnish researchers, they will be used here to avoid unnecessary confusion. These animals were selected for alcohol preference through a breeding program of more than 20 generations. Eriksson (13) conducted a genetic analysis of the AA and ANA lines and concluded that alcohol consumption is a polygenic, additive trait and that its heritability is low. The fact that the selection process required many generations to achieve separation of the two lines also indicates that the trait is complex and polygenic.

Eriksson is not the only investigator to breed rats successfully for differences in alcohol consumption. For example, Mardones (14) bred for alcohol preference by starting with a single mating pair of high-preferring and a single pair of low-preferring rats. Succeeding generations were obtained by brother-sister matings to produce inbred strains that exhibit a clear-cut difference in alcohol preference. Eriksson (5) points out that the approach used by Mardones has a major deficiency in that there is a strong possibility that any other strain differences were determined largely by the original mating pairs and are merely coincidental (or perhaps dependent on only one usually strong metabolic factor). This is essentially the same caution expressed by McClearn (1) with respect to the interpretation of data derived from inbred strains.

An interesting approach to the separation of genetic and environmental influences on alcohol preference was used by Randall and Lester (15). These investigators placed weanling mice of the C57BL (alcohol-preferring) strain with DBA (alcohol-avoiding) adults and vice versa. A choice of alcohol or water was available for seven weeks, at which time all mice were individually tested for alcohol preference. The C57BL offspring housed in a "non-drinking" environment drank about half as much alcohol as their siblings who were left with alcohol-preferring adults. Similarly, the normally alcohol-avoiding DBA offspring that were housed with "drinkers" drank almost twice as much alcohol as their siblings who were left with DBA adults. In spite of these marked changes in drinking behavior, the strain difference persisted, i.e., the "adopted" C57BL mice still drank more alcohol than the "adopted" DBA

mice. Somewhat different results were obtained when fertilized ova were transferred between alcohol-preferring and alcohol-avoiding mothers (16). In that study, C57BL offspring nurtured by DBA surrogate mothers actually increased their alcohol intake in spite of being born and raised by an alcohol-avoiding adult. DBA offspring born of C57BL surrogate mothers also drank more alcohol than DBA mice raised by DBA mothers.

Most recently, McClearn and Anderson (17) initiated a selective breeding program in mice for an alcohol consumption behavior which they called acceptance (see definitions). The lines have separated at the 10th generation. Calculated heritability is 0.15 in the high line and 0.28 in the low line. The gradual separation of the lines suggests that alcohol acceptance, like preference, is influenced by more than one gene. In addition, these investigators conducted tests to determine whether alcohol acceptance and alcohol preference are identical behaviors. Correlations were calculated among the following measures: (1) alcohol preference, as measured by the standard two-bottle choice method; (2) alcohol acceptance with thirst motivation; and (3) alcohol acceptance without thirst motivation. Although correlations between alcohol preference and the two acceptance measures were statistically significant, they were low. This observation led McClearn and Anderson to suggest that different methods of assessing alcohol consumption are not equivalent and that investigators studying mechanisms underlying alcohol consumption must be cautious in generalizing their results.

#### *Sex Differences in Alcohol Preference*

An influence of heredity on alcohol preference has also been seen when drinking behaviors of the two sexes are compared. Eriksson and Pikkarainen (9,18) noted a significantly greater preference for alcohol in female than in male C57BL mice. No sex difference was observed in the alcohol-avoiding DBA strain. Eriksson and Malmstrom (19) reported that female albino rats consume more alcohol than do males, and Eriksson (20) found that females of both the AA (high-preferring) and ANA (low-preferring) rat lines consume more alcohol than their male counterparts. Similarly, Brewster (21) observed a greater preference for alcohol in females than in males of the Maudsley Reactive and Maudsley Non-Reactive rat strains. Russell and Stern (22) noted a greater preference in females of the Wistar, Hooded, Tryon Maze Bright and Tryon Maze Dull rat strains. Taken together, these data argue strongly that, at least in laboratory mice and rats, female animals manifest a significantly greater preference for alcohol than do males.



### *Relationships Between Preference and Other Measures*

While genetic influences on alcohol preference are of interest in themselves, these demonstrated effects are of value in testing hypotheses concerning the underlying biochemical and physiological mechanisms which might account for differing preferences for alcohol. The genetic method has already been used to generate and test a few such hypotheses. Specifically, the roles of differential ethanol or acetaldehyde metabolism of neurotransmitter function have received the greatest attention.

**Alcohol Dehydrogenase.** Several studies have suggested that mouse strains with higher alcohol preference have greater hepatic alcohol dehydrogenase (ADH) activity as determined *in vitro*. One of the first suggestions that hepatic ADH may influence alcohol preference came from the studies of Rodgers *et al.* (6), who found a positive relationship between alcohol preference and *in vitro* hepatic ADH activity. Other studies (8,9,18) have confirmed this finding. However, the precise manner in which an elevated ADH activity influences alcohol preference remains a question. Schlesinger (23) observed that the alcohol-preferring C57BL strain, which has high hepatic ADH activity, attains blood alcohol concentrations differing only slightly from those of the alcohol-avoiding DBA strain following administration of the same dose of alcohol.

Koivula *et al.* (24), using Eriksson's AA and ANA lines, obtained evidence suggesting that alcohol preference is not directly related to hepatic ADH activity in rats. These investigators detected greater ADH activity in both sexes of the low-preferring ANA line than in the AA animals. Ethanol disappearance from the blood of AA females is faster than in ANA females, but there is no difference in disappearance rate between AA and ANA males (25). Thus, the data obtained from rats and mice are not in agreement. The mouse data suggest that alcohol preference increases with an increase in hepatic ADH activity, whereas the rat data suggest that the opposite situation may pertain. This discrepancy could be accounted for by assuming that the amount of ADH is not the rate-limiting factor in ethanol metabolism *in vivo*. Apparent relationships between ADH activity and ethanol preference or metabolism may be entirely coincidental.

One way to further test an hypothesized relationship between alcohol preference and hepatic ADH activity is to calculate correlations between the two variables in hybrid generations that are obtained by crossing two inbred strains that are known to differ in preference. The F2 generation provides a suitable group for testing correlations in that

segregation of genes, and therefore of genetically influenced traits, should have occurred. Only if genes which control the two traits are on the same chromosome may a spuriously high correlation be found between ADH activity and alcohol preference.

Another way to test an hypothesized relationship between behavior and biochemistry is to calculate correlations in a heterogeneous stock of animals (see definitions). For purposes of such genetic analyses, these animals can be used in the same way as an F2 generation, and they display even greater genetic variability. McClearn (26) tested the correlation between alcohol preference and hepatic ADH activity in an F2 generation obtained from a C57BL x DBA cross and in HS/Ibg mice (a heterogeneous stock derived from the crossing of eight inbred strains). No significant correlation was seen between preference and ADH activity in the F2 animals, while a low but significant correlation (.29) was found when the HS data were analyzed. These results may have been influenced by the fact that the F2 sample was very small (20 animals) in comparison with the larger number of animals (60) used in the HS study. Had a higher correlation been found between alcohol consumption and enzyme activity in these genetically heterogeneous groups, stronger evidence for a role of ADH activity in preference would have been obtained. Nevertheless, the correlation of .29 appears to be meaningful, and it indicates that the magnitude of the genetic influence on ADH activity is much smaller than might have been concluded on the basis of the earlier strain comparisons. Again, these results illustrate the potential hazards of inferring relationships between characteristics from their association in inbred strains. Even though relationships observed when inbred strains are compared provide only suggestive evidence for a causal connection between a given biochemical mechanism and some behavior, this approach has been utilized far more often than the more accurate method of examining correlations in genetically heterogeneous populations.

*Aldehyde Dehydrogenase.* The data are less ambiguous with respect to the second step in alcohol metabolism. Ethanol is converted by alcohol dehydrogenase to acetaldehyde, which is subsequently converted by hepatic aldehyde dehydrogenase (ALDH) to acetate (see definitions of biochemical pathways). One of the first observations which suggested that acetaldehyde levels may influence alcohol preference came from the studies of Schlesinger et al. (27), who noted that C57BL mice accumulate considerably less acetaldehyde in the blood following ethanol administration than do mice of the DBA strain. Furthermore, these investigators found that disulfiram (Antabuse), an inhibitor of ALDH, served to elevate blood

acetaldehyde and caused a significant decrease in alcohol preference in the C57BL strain. Additional evidence in support of the acetaldehyde hypothesis was reported by Sheppard et al. (28). When hepatic ALDH activity and alcohol preference were measured in the C57BL and DBA strains and in their F1 offspring, the C57BL's exhibited significantly greater ALDH activity than the DBA's and the F1 generation was intermediate to the parents in both preference and enzyme activity. Eriksson and Pikkarainen (9) examined alcohol preference and hepatic aldehyde oxidizing capacity in C57BL's and CBA's (similar to DBA's in that they are alcohol-avoiding) and in F2 offspring of a C56BL x CBA cross. Their data do not support the findings of Sheppard et al. (28), since no differences in liver cytosolic ALDH activity were detected among the groups. These results should be viewed cautiously, however, because recent data (29,30,31,32) suggest that cytosolic ALDH is of less importance with respect to total acetaldehyde metabolism than are the mitochondrial enzymes. Mitochondrial ALDH activity was not determined in the study by Eriksson and Pikkarainen (9).

It has previously been noted that females of the C57BL strain show a significantly greater preference for alcohol than do males (9,18). When Redmond and Cohen (33) compared acetaldehyde exhalation following ethanol injection in C57BL males and females, they found that males exhaled significantly more acetaldehyde. This finding suggests that the sex difference in alcohol preference in the C57BL strain may be related to differing acetaldehyde levels in males and females. A study by Koe and Tennen (34) demonstrated that butyraldoxime inhibited liver ALDH *in vivo*, increased blood acetaldehyde levels after ethanol treatment, and reduced alcohol consumption by C57BL mice.

Studies similar to those of Sheppard et al. (28) with mice have been carried out with the AA and ANA rats (24). ALDH activity was assayed with 0.5 mM acetaldehyde in various liver fractions of these animals. No difference was found in the enzyme activity in the mitochondrial fraction, although activity in the soluble fraction was greater in the AA line. This concentration of acetaldehyde approximates the concentration in the liver during ethanol oxidation (35). Eriksson (25) detected lower acetaldehyde concentrations in ethanol-perfused livers from AA rats than in those from ANA rats. A greater mitochondrial and microsomal ALDH activity was found by Koivula et al. (24) in the AA than in the ANA line when a high-concentration (4.5 mM) propionaldehyde was used as substrate. The use of this concentration of aldehyde measures the total amount of ALDH that is present. These data suggest that the AA and ANA lines have similar activities of the

low- $K_m$  mitochondrial enzyme (measured with 0.5 mM acetaldehyde), while the activity of the high- $K_m$  mitochondrial enzyme (measured with 4.5 mM propionaldehyde) is greater in the AA line. When acetaldehyde (0.4 mM) metabolism was measured in a homogenate system, the rate of acetaldehyde elimination was slightly greater in the AA animals. These findings may be compared to reports from a number of laboratories (29,30,31, 32) that the low- $K_m$  enzyme in rat liver mitochondria is primarily responsible for acetaldehyde metabolism. Collectively, these data indicate that higher  $K_m$  enzymes may play a role in the control of blood acetaldehyde level. A factor not considered here, however, is the possibility that ALDH activity in other tissues may be of importance and may be different in the AA and ANA lines. In any event, it appears that high blood acetaldehyde is a deterrent to alcohol consumption in rats and mice. This was confirmed serendipitously by the finding that a change in diet caused a marked reduction in ethanol preference and a corresponding decrease in rat liver acetaldehyde metabolism (35). Added confirmation that acetaldehyde is a deterrent to alcohol consumption comes from the observation that a diet low in protein and high in carbohydrates results in elevated blood acetaldehyde, a decrease in rate of ethanol metabolism, and reduced alcohol consumption (36).

Another means of altering acetaldehyde metabolism has been to utilize the induction of a liver cytosolic ALDH (37). This induction is brought about by phenobarbital, it is known to be controlled by a single gene, it is observed only in the liver cytosol, and it is dependent upon genotype in certain rat strains (38). A rat liver cytosolic enzyme, called the  $\phi$  enzyme, is induced from ten to thirty-fold by phenobarbital treatment in homozygous reactor (RR) animals; animals that do not show induction following phenobarbital treatment are designated rr. The  $\phi$  enzyme is present in non-treated animals (39), but the genetically determined induction effect is present only in some rat strains such as Long-Evans, Sprague-Dawley, Wistar and ACIF (38). The induced enzyme has a high  $K_m$  (mmolar range) for acetaldehyde, and, when induced, it does alter blood acetaldehyde in ethanol-treated animals but not to a degree consistent with the large increase in enzyme activity (40,41). Similarly, the metabolism of dopamine or norepinephrine in rat liver slices is not markedly altered by induction of this enzyme (42).

Induction of another ALDH isozyme ( $\tau$  enzyme) in the cytosol of rat liver by tetrachlorodibenzo-p-dioxin (TCDD) has recently been reported (43). In this case, a genetic effect has not been found. The induction is approximately a hundred-fold, but the  $K_m$  for acetaldehyde is even higher than that of the phenobarbital-induced enzyme (44). Petersen et al. (40)

found that even such a large induction had no effect on blood acetaldehyde after ethanol treatment or on dopamine metabolism in rat liver slices. Basic principles of enzymology adequately explain such results, since it is known that liver acetaldehyde levels during ethanol metabolism rarely exceed 0.5 mM (45). From the Michaelis-Menten equation ( $v/V_{\max} = S/(K_m + S)$ ) and the  $K_m$  values for acetaldehyde of the  $\phi$  enzyme (2.7 mM) and the  $\tau$  enzyme (22 mM), one can easily calculate that the  $v/V_{\max}$  ratio for the  $\phi$  enzyme will be 0.16 (or 16% of  $V_{\max}$ ), while that for the  $\tau$  enzyme will be 0.019 (or 1.9% of  $V_{\max}$ ). In the case of the  $\phi$  enzyme, operating at 16% of its  $V_{\max}$  (which is achieved at 0.5 mM acetaldehyde), a thirty-fold induction should increase the cytosolic contribution to acetaldehyde metabolism by a factor of 4.8. If the cytosol contributes 15% of the total acetaldehyde oxidizing capacity of rat liver, the total increase in this capacity will be 0.72 times. In other words, a gram of liver in which the  $\phi$  enzyme has been induced will be operating at 172% of the capacity of a gram of control liver. When the  $\tau$  enzyme is induced a hundred-fold, the contribution of the cytosol to acetaldehyde (0.5 mM) oxidation will increase by a factor of 1.9. Thus, liver in which the  $\tau$  enzyme has been induced will be operating at 129% compared to control liver. These calculations agree with our finding that induction of the  $\phi$  enzyme decreased blood acetaldehyde concentration, while induction of the  $\tau$  enzyme was without effect. Eriksson et al. (41) obtained similar results following induction of the  $\phi$  enzyme, although these authors found no difference in acetaldehyde oxidation rate per gram liver *in vivo* when a correction for blood flow through the liver and ethanol oxidation rate was taken into account.

Marcelos et al. (46) tested AA and ANA rats for alcohol preference thirty days after treatment with phenobarbital. Although both lines manifest a slit induction of cytosolic ALDH by phenobarbital, the treatment had no effect on preference in either line. Also, no effect of treatment on preference was found in two other rat strains, one which showed an induction of cytosolic ALDH and one which did not. It should be emphasized that this study does not prove that an increase in cytosolic ALDH activity has no influence on alcohol preference, because preference was not measured during the time when enzyme activity was increased. The findings of Marselos et al. demonstrate only that alcohol preference is not influenced by inducibility of ALDH activity. These results are not surprising, since there is no reason to believe that potential for induction, a single-gene effect, should be related to a polygenically determined behavior such as alcohol preference. Nevertheless, studies involving enzyme induction may be useful in examining the effects of acetaldehyde on preference.

Marselos and Pietikainen (47) conducted a direct test of the effect of ALDH induction on preference by using DDT to induce rat liver cytosolic ALDH. No difference in alcohol preference between rats that showed induction (RR) and those that did not (rr) was observed under these conditions. Results from our laboratory (44) indicate that there are a number of enzymes induced by phenobarbital, as well as the specific enzyme induced by TCDD. There is no information as to which of these multiple isozymes is induced by DDT; if it is an enzyme with a very high  $K_m$ , no effect on blood acetaldehyde would be expected. Blood acetaldehyde concentrations were not measured by Marselos and Pietikainen (47).

It must be pointed out that blood acetaldehyde is customarily measured after administration of rather large doses of ethanol, whereas the amount consumed by animals in an alcohol preference study is relatively small. Nevertheless, an overall view of the accumulated data does support the hypothesis that alcohol preference is influenced by the concentration of acetaldehyde in the blood. Although this variable appears to be controlled principally by the low- $K_m$  mitochondrial ALDH, activity of high  $K_m$  isozymes, such as the  $\phi$  enzyme, may be critical in the fine control of blood acetaldehyde concentration.

*Biogenic Amines.* Another major hypothesis concerning underlying mechanisms which might account for differences in alcohol preference arose from the observation that drugs which influence brain serotonin level also appear to have an effect upon preference. However, no clear picture has emerged from the results of studies in this area. For example, Hill (48) found a reduction in alcohol consumption following intraventricular injection of serotonin, and similar results were obtained when 5-hydroxytryptophane (the metabolic precursor of serotonin) was administered (49,50). On the other hand, drugs such as parachlorophenylalanine and parachloroamphetamine, which decrease brain serotonin level, have also been observed to reduce alcohol preference (51,52,53), although some investigators (49) have found an increase in preference following treatment. Sanders et al. (53) observed that pargyline, an inhibitor of monoamine oxidase, also serves to decrease alcohol preference. These inconsistencies may be explained by the suggestion of Nachman et al. (54) that those drugs which decrease preference may be acting as an aversive stimulus. Dembiec et al. (55) found that pargyline inhibits hepatic ALDH and causes an elevation in blood acetaldehyde level. Peterson et al. (40) observed that pargyline inhibits only the low- $K_m$  ALDH found in liver mitochondria. This suggests that pargyline may be acting in a manner similar to disulfiram. Results of studies recently completed in our

laboratories support this suggestion. In these studies, utilizing C57BL/1bg mice, a decrease in alcohol preference was detected following administration of either pargyline or Lilly 51641 (another monoamine oxidase inhibitor), while nialamide, which inhibits monoamine oxidase but not ALDH, did not influence preference. Dembiec et al. (55) noted that pargyline and Lilly 51641 elevate blood acetaldehyde and that nialamide has a minimal effect. Thus, inconsistencies in these data could be explained by the possibility that acetaldehyde may be acting as an aversive stimulus. Ambiguities in the results of studies intended to examine effects of alterations in brain amine content on alcohol preference may be attributable to such confounding effects.

Because genetic analyses do not require the use of drugs, which generally have a multitude of actions, such problems are less likely to arise. Unfortunately, genetic methods have seldom been used in studies of the relationship between neurotransmitters and alcohol preference. Ahtee and Eriksson (56) measured whole brain concentrations of serotonin and 5-hydroxyindole acetic acid (5HIAA) in AA and ANA rats. The concentrations of both of these compounds were 15-20% higher in the AA line, but the difference was not statistically significant. When 5HIAA removal from the brain was inhibited by probenecid, the 5HIAA content of AA brain was significantly greater than that of ANA brain. This finding indicates that turnover, rather than static levels of amines, may play an important role in alcohol preference. Furthermore, ethanol consumed in the drinking water elevated serotonin in the AA but not in the ANA line. This implies that there is an interaction between alcohol treatment and one or more of the processes which influence serotonin level (i.e., rate of synthesis, release, reuptake or metabolism). Recently, the same investigators (57) measured brain catecholamine content in the AA and ANA animals. A greater concentration of dopamine, but not norepinephrine, was found in the AA line.

Pickett and Collins (10) examined whole brain concentrations of serotonin in the alcohol-preferring C57BL and the alcohol-avoiding DBA mouse strains and in their F1 and F2 offspring. There was no difference in serotonin content between the parental strains, and there was no apparent relationship between serotonin level and alcohol preference in the F2 generation. These data are in agreement with the findings of Ahtee and Eriksson (56) in suggesting that alcohol preference is not influenced by static brain serotonin levels. If this biochemical variable had an influence on preference, a clear-cut relationship should have been observed in the F2 generation in the study by Pickett and Collins (10). Correlational studies using other genetically heterogeneous populations

would provide a more rigorous test of the hypothesis that neurotransmitter function is involved in alcohol preference.

### Initial Sensitivity to Alcohol

In comparison with alcohol preference research, far fewer studies indicate that initial central nervous system sensitivity to alcohol is influenced by genetic factors. Most of the studies have utilized inbred or selectively bred mice. As in preference research, the conclusion that there is a genetic influence on sensitivity is based upon results showing that different inbred strains respond differently to alcohol and that differential sensitivity between lines may be achieved by a selective breeding program.

A study from McClearn's research group (58) provided information as to the cause of differing sensitivities to alcohol. In this study, mice of the C57BL and BALB/cCr<sub>g</sub>l strains were injected with a hypnotic dose of ethanol and duration of loss of the righting reflex (alcohol-induced "sleep time") was measured. Animals of the BALB strain slept over 3.5 times as long as did mice of the C57BL strain. A portion of the difference in sleep time could be explained by the observation that the C57BL strain regained the righting response ("time of awakening") at a blood alcohol concentration considerably higher than that seen in the BALB strain at time of awakening. These data, and results of an earlier preliminary study (59), indicate the genotype influences neuronal sensitivity to alcohol.

Evidence which suggests that enzymes involved in alcohol metabolism influence duration of alcohol-induced sleep time has come from several studies. Belknap et al. (60) examined the correlation between duration of sleep time and hepatic ADH and ALDH activities in the heterogeneous stock of mice (HS/Ibg) developed by McClearn (61). A correlation of -0.57 was found between ADH activity/gram body weight and sleep time, accounting for approximately one-third of the variance in sleep-time scores. A smaller correlation (-0.39) between ALDH activity/gram body weight and sleep time was obtained. Genetic influences on sleep time can, therefore, be explained at least in part, by differential activities of those enzymes which are of primary importance in alcohol metabolism. Damjanovich and MacInnes (62) studied the effect of genotype on fall time (the time from ethanol injection to loss of ability to cling to a wire mesh) and on sleep time using three inbred mouse strains (C57BL/6J, DBA/2J and BALB/cJ). The results showed differences in fall time, which could be explained by differential rates of alcohol absorption; there were also differences in sleep time, which the authors suggested might be explained, at least in part, by differing rates of alcohol metabolism.



Several other studies, also utilizing inbred mouse strains, have added to the evidence that genotype interacts with alcohol to influence behavior. For example, Randall et al. (63) examined the effect of various alcohol doses (0.75, 1.50 and 2.25 grams/kilogram body weight) on locomotor activity in C57BL and BALB/cJ mice. The C57BL strain showed a dose-dependent decrease in locomotor activity, whereas the BALB/cJ strain showed an increase in activity over this dose range. These data may be explained by the hypothesis that C57BL mice are more sensitive to the depressant action of alcohol, while BALB mice are more sensitive to the activating effect of this drug. Comparison of these findings with those of the Kakihana et al. study (58) suggest that the factors which result in a decrease in locomotor activity may not be the same as those involved in hypnosis, since the previous investigation found less neuronal sensitivity to the hypnotic actions of alcohol in C57BL than in BALB mice.

Alcohol may also influence learning and memory, and the effects seem to be related to genotype. This suggestion is based upon the results of a study by MacInnes and Uphouse (64), who measured the effects of several doses of alcohol (ranging from 0.5 to 3.0 grams/kilogram) on the acquisition and retention of a passive-avoidance task in C57BL and DBA mice and in the F1 generation produced by crossing these two strains. In the acquisition of the task, C57BL mice were relatively unaffected by doses of alcohol that seriously interfered with the performance of both DBA and F1 animals. On the other hand, when retention was measured the following day, the F1's showed better retention after higher doses during acquisition than either parental strain.

Perhaps the best evidence indicating the initial sensitivity to alcohol is under genetic control comes from the studies of McClearn and Kakihana (65). These investigators selectively bred mice for long or short sleep time following a hypnotic dose of ethanol (4.1 gram/kilogram). The foundation population was the heterogeneous stock of mice (HS/Ibg) developed by McClearn (61). After eighteen generations of selective breeding (see definitions), there was virtually no overlap in sleep time between the long-sleep (LS) and short-sleep (SS) lines. The mean sleep time of the SS line was eleven minutes, and that of the LS line was approximately 140 minutes. Heston et al. (66) measured the activity of hepatic ADH and ALDH, the rate of *in vivo* ethanol elimination, and the ED50 for loss of the righting response in the two lines. No differences in ADH or ALDH activity were detected, and *in vivo* rates of ethanol metabolism were similar in the two lines. ED50 for loss of the righting response, however, was nearly two times as great in the SS as in the LS line. These data suggest that the two lines differ principally in neuronal sensitivity to the

hypnotic effects of ethanol. Erwin et al. (67) also noted that the lines differ in sensitivity to hypnotic doses of several alcohols, while they detected no differences in sleep time following administration of pentobarbital, diethylether, chloral hydrate, paraldehyde or trichloroethanol. Thus, these animals may be very valuable in ascertaining the specific mechanisms by which alcohol elicits its hypnotic effects. For example, they have already proved to be of value in testing one of the more controversial hypotheses concerning alcohol's mechanism of action. Church et al. (68) assessed the sensitivity of the LS and SS mice to intraventricular injection of salsolinol, the tetrahydroisoquinoline alkaloid which Cohen and Collins (69) have suggested may form *in vivo* as a consequence of the condensation of acetaldehyde with dopamine. The LS mice were found to be nearly twice as sensitive as the SS to the effects of salsolinol as measured by depression of locomotor activity. Because the LS and SS lines differ in sensitivity to alcohols but not to other hypnotics (67), these data support the hypothesis that salsolinol, or related compounds, may play a role in the depressant action of alcohol.

The hypothesis that alcohol's depressant effect is influenced by interactions with neurotransmitters has also been tested using LS and SS mice. Chan (70) detected significantly higher GABA levels in cerebral cortex and pons-medulla of LS than SS mice, whereas no difference in brain glutamic acid content was observed. Collins et al. (71) found significantly greater concentrations of both dopamine and norepinephrine in the brain of SS mice. Administration of ethanol (4.1 gram/kilogram) decreased dopamine turnover in both lines, but to a significantly greater extent in the LS. It should be emphasized, however, that finding differences between selected lines does not provide unambiguous evidence to support a hypothesis. The hypothesis should be further tested by examining the correlation between the behavioral trait and the mechanism in question in a second or perhaps a third generation derived by crossing the two lines.

One other point should be made concerning the LS and SS mice. These animals were selectively bred for differential sensitivity to the hypnotic effects of alcohol, and there is no reason to expect that they would differ in the same fashion with respect to other actions of this drug. In fact, Sanders (72) recently reported that the LS mice are less sensitive than the SS to the activating effects of low doses of ethanol. Interestingly, Sanders also found that the LS animals are less sensitive to the activating effects of pentobarbital.

An overall view of the above studies indicates that the results have tended to vary as a function of the behavior that is measured and the alcohol dose that is administered. These observations suggest that alcohol may exert its influences via

a number of different mechanisms. Thus, an understanding of how alcohol affects a specific behavior may be best obtained by utilizing animals which differ in sensitivity to alcohol as measured by that particular behavior. Furthermore, since many differences appear to be dose-dependent, it is probably necessary to use various alcohol doses in testing any hypothesis.

The observations that inbred strains of mice differ in sensitivity to various effects of alcohol and that selective breeding for alcohol's hypnotic actions has been successful are strong arguments in favor of a genetic influence. However, only a relatively few studies have utilized a genetic approach in attempts to determine the mechanisms which underlie differences in sensitivity. Hopefully, further application of genetic methods will provide critical evidence as to biochemical and physiological mechanisms which might account for differing initial sensitivities to alcohol.

#### Physical Dependence Upon Alcohol

Only two studies have attempted to determine whether the development of physical dependence upon alcohol is influenced by genetic factors. In the first of these investigations, Goldstein (73) carried out a modified selective breeding program. Male and female Swiss-Webster mice were subjected to two cycles of intoxication and withdrawal. Intoxication was achieved by Goldstein's established procedure of injecting animals with pyrazole to inhibit alcohol metabolism and administering alcohol by inhalation. When withdrawal was evaluated by scoring the convulsions elicited by handling, a wide range of withdrawal scores was observed. Two pairs of high-scoring and two pairs of low-scoring mice were bred, and their offspring were tested for severity of withdrawal according to the same procedure. A significant difference in mean withdrawal score between offspring of high-scoring and low-scoring parents was evident even in this first generation. A second generation was then selectively bred, and the between-line difference was greater than that observed in the first generation. Additional evidence of a genetic effect on withdrawal severity was found in this study in that male mice in all three generations exhibited a more marked withdrawal syndrome than did females. Goldstein suggests that this sex difference may be explained by the fact that the males attained higher blood alcohol concentrations. This observation implies that the sex difference in withdrawal severity may be dependent upon blood alcohol level rather than differential central nervous system sensitivity to alcohol. If the development of physical dependence upon alcohol is facilitated by maintenance of higher blood alcohol concentrations for a longer period of time, males, which

metabolize alcohol more slowly than females, should become dependent more rapidly.

One additional observation should be made concerning this study. The rapid separation between the lines obtained by the selective breeding procedure suggests that withdrawal severity may be influenced by only a few genes or, if many genes are involved, that only a few are of major importance. In any event, these results clearly indicate that withdrawal severity is influenced by genetic factors.

The only other published study of genetic influences on withdrawal was carried out by Goldstein and Xakihana (74). When these investigators used Goldstein's standard procedure to assess severity of withdrawal in LS and SS mice, the SS animals showed a more intense withdrawal reaction. The investigators then attempted to ascertain whether this difference in withdrawal severity was related to differential sleep time by comparing sleep-time and withdrawal scores in mice of the HS/Ibg stock. Because no correlation between these measures was found, it was concluded that no relationship exists between sleep time and withdrawal severity. However, it should be pointed out that the LS and SS lines differ only in central nervous system sensitivity to alcohol and have similar rates of alcohol metabolism (66). The HS mice, on the other hand, undoubtedly vary in both neuronal sensitivity and metabolism rate. It is possible that the SS mice, which have less initial sensitivity (greater tolerance) to alcohol, show a more marked withdrawal syndrome than the LS mice because those neurochemical factors which contribute to greater tolerance also contribute to the development of physical dependence. A study utilizing HS mice and designed to measure the correlation between central nervous system sensitivity, as indicated by blood alcohol level at time of regaining the righting response, and withdrawal severity might determine whether a greater initial tolerance to alcohol facilitates dependence development.

#### LOWER ORGANISMS AS MODELS

The long history of the utility of *Drosophila melanogaster* (fruit flies) in genetic research is well known. Some of these studies are relevant to our discussion of alcohol-related behaviors. For example, McKenzie and Parsons (75) reported that flies derived from a vineyard or from a wine cellar were more resistant to the effects of alcohol than those derived from an area remote from the vineyard. Although the presence of ADH in normal amounts is necessary if the flies are to use ethanol as a carbon source (76), the difference in resistance did not appear to be related to ADH level. Another species, *Drosophila simulans*, was never found in the wine cellar and might,

therefore, be called a non-preferring species. Actually, *D. simulans* does reject media containing 9% alcohol for purposes of oviposition, and this species is also more sensitive to alcohol, both as adults and as larvae, than is *D. melanogaster* (75,77). While fruit flies are even further removed from the problems of human alcoholism than are laboratory rodents, their short generation time and relatively simple genetic structure might make them an interesting model for further studies on the pharmacogenics of alcoholism.

Genetic influences on responses to alcohol have also been studied in microorganisms. The interest in yeast is obvious, from a commercial as well as a scientific viewpoint, and it has a long history that extends back to Pasteur (78,79). Even bacteria (80,81) and a fungus (82) have not been ignored in the study of the effects of alcohol. Of particular interest is the study by Fried and Novick (81) utilizing mutants of *Escherichia coli* which are resistant to ethanol. The genetic alteration appears to affect the cell membrane. As the authors point out, this may provide a useful tool for study of the physical biochemistry of the action of ethanol on cell membranes in general.

#### HUMAN STUDIES

A number of investigators have observed genetic differences in response to ethanol in humans. One of the most frequently studied of these genetically influenced responses is the flush reaction which occurs in Orientals (83,84) and American Indians (85,86). An unpleasant flush reaction occurs in the blush region almost immediately upon ingestion or intravenous administration of ethanol. Wolff (86) has postulated that the response is due to a genetically influenced variation in responsiveness of the vasomotor system. Another possible explanation involves the metabolism of ethanol. During ethanol metabolism, the concentration of NAD drops and there is a corresponding rise in NADH level. It is now widely accepted that, given a normal amount of ADH, the rate-limiting step in ethanol metabolism lies in the reoxidation of NADH to NAD. Under normal conditions, the absolute amount of ADH is not the rate-limiting factor. However, some individuals, notably a large percentage of Japanese, have an "atypical" liver ADH (87,88). Because this enzyme is much more active than the "typical" enzyme, there may be an initial "burst" of ethanol metabolism before the NAD/NADH ratio has been markedly lowered by ethanol, and this phenomenon may overwhelm the ability of the liver to metabolize the resultant acetaldehyde. After a few minutes, the NAD/NADH ratio would drop and reoxidation of NADH and NAD would become rate limiting. This

suggestion is supported by the fact that individuals with the "atypical" enzyme do not carry out the overall metabolism of ethanol any more rapidly than do those with the "typical" liver ADH (89,90). Since it is well known that acetaldehyde causes unpleasant reactions, as many disulfiram (Antabuse) users will attest, this product of ethanol oxidation may be responsible for the flush seen in Orientals who ingest ethanol.

The supposed Asiatic origin of the American Indian (85, 86) leads to the speculation that this group may also show a high incidence of the "atypical" liver ADH. A recent paper by Bennion and Li (85), however, casts doubt on this hypothesis. While the flush reaction to ethanol does occur in Indians (86), liver biopsy of seven individuals failed to demonstrate the presence of the "atypical" ADH in any case. Unfortunately, the individuals on whom the biopsies were performed were not tested for the flush reaction, so the relationship between the two variables could not be determined. If it is true that the "atypical" enzyme is involved in the flush reaction, and that this reaction in turn is important in rejection of alcohol by the Japanese, then a paradox arises. The incidence of alcoholism among Japanese is comparatively low, but there is a high incidence among American Indians. It has been suggested (88) that the increased acetaldehyde may in one case be a deterrent to excessive alcohol intake, while it may lead to greater addiction liability in a different culture. (Also, see material presented earlier in this chapter and reference 92 for a discussion of the role of acetaldehyde in the actions of ethanol.)

The question of rate of alcohol metabolism in Indians as compared to Caucasians has been the subject of considerable controversy. Fenna et al. (93) found that Canadian Indians and Eskimos metabolized alcohol more slowly than Caucasians and attributed this difference to racial variation. Their interpretation has been challenged on theoretical grounds by Lieber (94). The study of Bennion and Li (85) found no difference in rate of ethanol metabolism between Southwestern Indians and Caucasians. Despite this controversy, there is no doubt that genetic differences in overall alcohol metabolism do exist among humans. Vessel et al. (95) used the twin method, a common procedure for distinguishing between genetic and environmental effects, to investigate factors affecting ethanol metabolism. A genetic influence was suggested by the finding that dizygotic twins exhibited different rates of metabolism, while monozygotic twins did not. It is possible, however, that an effect due to differential volumes of distribution may also have been involved in these results.

It should be noted that at least two precautions are necessary, but not always taken, in studies of human response to

ethanol. First, rate of ethanol metabolism must be expressed in terms of body weight and corrected for volume of distribution. Second, it is important to distinguish between genetic and environmental influences when ethnic groups are compared. While it is relatively easy to do this in twin studies by establishing the zygosity of twin pairs, it may be nearly impossible to determine genotypes of individuals in different ethnic groups. There is no single, objective method of verifying genotype, and family records are seldom detailed enough to indicate the genetic background of an individual.

Much work has been carried out in an attempt to find a genetic marker associated with alcoholism. Needless to say, such a marker would be most useful in counseling and in prevention programs. Positive associations between certain blood groups (A, notably) or color blindness and alcoholism have been reported (see reference 5 for a discussion). More recently, a relationship between other blood groups (S and D, as well as complement C3) and alcoholism has been found (96).

Many studies have shown that alcoholism in humans has a genetic component (97,98,99,100,101). Furthermore, many studies have demonstrated an association between alcoholism and serological markers (e.g., 96). However, the results of these genetic investigations have not provided a biochemical theory to explain human variability in response to alcohol. The development of such a theory, rather than continued confirmation of the fact that there is a genetic influence on human alcoholism, would seem to be a more profitable focus for future research. To date, only the occurrence of an "atypical" ADH in some humans has provided a biochemical clue to human alcoholism and it has not yet been proven that this condition is predictive.

## SUMMARY

The literature cited in this review provides unequivocal evidence to support the notion that several alcohol-related behaviors are influenced by genetic factors. Our progress toward an understanding of biochemical mechanisms underlying preference for alcohol, initial sensitivity to alcohol, and the development of physical dependence upon alcohol has been facilitated by the use of animal models that differ genetically in one or more of these behaviors. For example, use of genetic methods has provided a great deal of evidence in support of the hypothesis that high blood acetaldehyde levels are aversive and result in decreased preference for alcohol. The results of studies with disulfiram (Antabuse) lead to the same conclusion. However, any study in which a drug is utilized must be interpreted with caution, since few, if any, drugs have only one effect on a living organism.

It is our hope that readers of this review will not only have been familiarized with the evidence that variability in alcohol-related behaviors is influenced by genetic factors, but will also have become acquainted with the use of genetic analysis as a means of testing hypotheses concerning the actions of alcohol and other drugs. This little-used technique, like the administration of a drug which serves to stimulate or inhibit a biochemical pathway or a physiological process, can be applied to the investigation of underlying mechanisms which might account for variations in a particular behavior. Genetic analyses have the advantage of being less susceptible to the confounding effects which may occur when drugs are used, while they often have the disadvantage of requiring more time and effort. Hopefully, a combination of approaches will provide the tools necessary to elucidate the mechanisms which underlie human alcoholism.

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## 8. A MECHANISM FOR ALTERED ADRENERGIC ACTIVITY IN ALCOHOLISM

Gerald Cohen

Mount Sinai School of Medicine, Department of Neurology, New York, New York, 10029.

### INTRODUCTION

The purpose of this manuscript is to describe a special interaction of the ethanol metabolite, acetaldehyde, with adrenergic systems. The term "adrenergic systems" is taken to comprise all catecholamine-containing nerve tracts, both in the central nervous system and in the peripheral sympathetic system; additionally, the adrenal medulla is included, as are certain of the clusters of interneurons in sympathetic ganglia (e.g. the dopamine-containing interneurons).

It has been established (1) that catecholamines within intact cells can condense spontaneously with acetaldehyde or with formaldehyde (a metabolite of methanol) to yield a group of ring-closed products which contain the 1,2,3,4-tetrahydroisoquinoline (TIQ) nucleus (Figure 1).



*Fig. 1. Route of synthesis of 6,7-dihydroxy-TIQs from catecholamines in adrenergic systems. Acetaldehyde,  $R_3 = \text{CH}_3$ ; formaldehyde,  $R_3 = \text{H}$ . Dopamine,  $R_1 = R_2 = \text{H}$ ; norepinephrine,  $R_1 = \text{OH}$ ,  $R_2 = \text{H}$ ; epinephrine,  $R_1 = \text{OH}$ ,  $R_2 = \text{CH}_3$ .*

These alkaloid products contain hydroxyl groups in the 6- and 7-positions, arising from the catechol portions of the corresponding catecholamines. A requirement for the ring-closure reaction to proceed at neutral pH is the presence of an activating hydroxyl group in the 3-position (the meta position) of



the catecholamine (2). The hydroxyl group in the corresponding 4-position (the para position) does not strongly influence the ring-closure reaction. Thus, m-tyrosine and m-tyramine will condense spontaneously, but the naturally-occurring p-tyrosine and p-tyramine will not. For purposes of organic synthesis under laboratory conditions, the reactions of phenylethylamines without activating meta hydroxyl groups can be catalyzed by heat and strong acid (3).

The formation of TIQs in the adrenal medulla during perfusion of isolated cow adrenal glands with acetaldehyde or formaldehyde has been studied in detail (1,4-7). There is evidence for biosynthesis of TIQs in the adrenals of methanol-intoxicated rats (8). Recently, Collins and Bigdeli (9) demonstrated the presence of one TIQ (salsolinol, formed by the condensation of dopamine with acetaldehyde) in the brains of rats treated with ethanol in combination with other drugs. Some studies have also been conducted with a purely *in vitro* system consisting of broken cell preparations (homogenates) of brain (10).

Because TIQs possess catecholamine-like structures, their interaction with adrenergic mechanisms is not unexpected (1). The numerous interactions of TIQs with adrenergic neurons were the subject of a recent review (11) (see also other articles in this volume). TIQs block uptake of catecholamines *in vitro* and *in vivo*. TIQs are themselves taken up and accumulated within the catecholamine-storage vesicles. They can be secreted from cells by calcium-dependent mechanisms. TIQs also appear to interact with catecholamine receptors: both agonist and antagonist actions have been noted. Some of these properties of TIQs are described in greater detail in this chapter.

#### *SYNTHESIS OF TIQ ALKALOIDS AND RELEASE FROM THE ADRENAL MEDULLA BY ACETYLCHOLINE*

Retrograde perfusion of the catecholamine-rich, cow adrenal gland with solutions of acetaldehyde or formaldehyde results in the synthesis of TIQ derivatives of epinephrine and norepinephrine. TIQ products within the adrenal medulla were observed routinely after perfusion with solutions containing 2.3 mM acetaldehyde (100 µg/ml) (1). Radiotracer studies with <sup>14</sup>C-acetaldehyde showed that small amounts of TIQ products were formed when 23 µM acetaldehyde (1 µg/ml) was used (12); this latter concentration is in the range seen in human subjects during ethanol intoxication (13). When a relatively high concentration of formaldehyde was used (33 mM; 1 mg/ml), total conversion of adrenal catecholamines to TIQs was observed (4); such large conversions were not achieved at correspondingly high concentrations of acetaldehyde. The

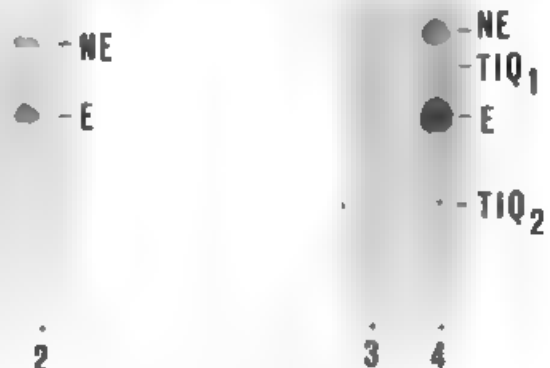
relatively greater extent of reaction of catecholamines with formaldehyde most probably results from a very much greater rate of reaction of formaldehyde compared to acetaldehyde (1).

The TIQs formed in the adrenal gland are bound in the catecholamine-storage vesicles (chromaffin granules) (14). Recent studies by Schneider (7) with  $^{14}\text{C}$ -acetaldehyde have shown that  $^{14}\text{C}$ -TIQs account for over 95% of the soluble  $^{14}\text{C}$  found in adrenal chromaffin granules. Other investigators (15) have established that release from the adrenal medulla takes place by exocytosis. In this process, which requires calcium ions, the chromaffin granules appear to fuse with the outer cell membrane and then they release their entire soluble contents, which include the catecholamines, ATP, dopamine beta-hydroxylase, and chromagranin (a binding protein). Because TIQs are constituents of the chromaffin granule in acetaldehyde-perfused glands, it appeared likely that stimulation of such glands would lead to the release of the TIQs along with the catecholamines. Studies were undertaken to test this possibility (5):

Paired glands were perfused for one hour with either Tyrode's solution (controls) or 23 mM acetaldehyde in Tyrode's solution (experimental glands). The relatively high concentration of acetaldehyde was used to ensure a reasonable yield of TIQs in order to facilitate analyses by thin-layer chromatography. The glands were rinsed by perfusion with fresh solution without acetaldehyde for an additional hour. Subsequently, stimulation by perfusion with Tyrode's solution containing 0.16 mM acetylcholine for two minutes, resulted in release of catecholamines from the control gland, and catecholamines plus TIQs from the experimental gland (Figure 2).

**Fig. 2.** Thin-layer chromatographic assays of  $\text{Al}(\text{OH})_3$ -purified extracts of the perfusates from intact cow adrenal glands. Thin-layer chromatography was run in an upward direction on plates composed of Silica Gel G; the solvent was sec-butanol: formic acid: water (15: 3: 2). Plates were

sprayed with  $\text{K}_3\text{Fe}(\text{CN})_6$  followed by  $\text{FeCl}_3$ , which produced the intensely-colored Prussian blue wherever reducing agents (catechols) were present on the plate. The method is sensitive to about 0.1  $\mu\text{g}$  catecholamine or TIQ. For further details, see reference 5. The figure shows paired adrenal glands before and during stimulation with 0.16 mM acetylcholine. Control gland, perfused with saline alone: (1) prior to and (2) during



stimulation with acetylcholine. Acetaldehyde-perfused gland: (3) prior to and (4) during stimulation with acetylcholine. For structures of TIQ<sub>1</sub> (norepinephrine condensed with acetaldehyde) and TIQ<sub>2</sub> (epinephrine condensed with acetaldehyde) refer to figure 1.

Similar observations were made with carbachol (carbamylcholine) as the secretagogue.

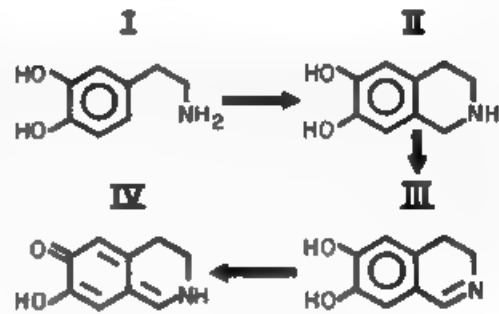
The secretion process for TIQs, like that for catecholamines, was dependent upon calcium ions. When glands were stimulated with carbachol in the absence of Ca<sup>2+</sup>, neither the catecholamines nor the TIQs were found in the effluents from the glands. Secretory responses were restored by replenishing Ca<sup>2+</sup> to the gland. No secretion of catecholamines or TIQs was evident in the presence of 0.1 mM tetracaine, an agent that prevents the inflow of Ca<sup>2+</sup> into stimulated glands. Thus, it appeared that the secretion of TIQs and catecholamines took place by the same process. In more recent studies, Rahwan et al. (6) confirmed and extended these observations; these investigators also noted that in the absence of Ca<sup>2+</sup>, acetaldehyde by itself evoked the release of catecholamines, while the TIQs were retained within the gland.

#### UPTAKE, STORAGE AND RELEASE OF TIQs BY PERIPHERAL SYMPATHETIC NERVES

The probability that TIQs would enter adrenergic nerve terminals of the brain was established by the observation that TIQs interfered with (blocked) the uptake of <sup>3</sup>H-catecholamines (16). More recently, blockade of <sup>3</sup>H-catecholamine uptake by TIQs and some other alkaloids was studied in greater detail (17,18). The results of these and other studies indicate that TIQ alkaloids and <sup>3</sup>H-catecholamines compete for transport sites on the axonal membrane. Studies with <sup>3</sup>H-labelled TIQs have shown that some TIQs are actively taken up and accumulated by adrenergic nerve terminals of the brain *in vitro* (16) and by sympathetic nerve terminals of the heart, iris and sub-maxillary gland of mice or rats *in vivo* (19).

In the studies described below, fluorescence microscopy was used to study the uptake, storage and release of TIQs by the peripheral sympathetic nerve plexus in the rat and mouse iris. Formaldehyde-derived TIQs are intermediates in the well-known method for the visualization of catecholamines in tissues by means of fluorescence microscopy (reviewed by Corrodi and Jonsson, [20]). In this method, tissues are heated with formaldehyde gas under carefully defined conditions of humidity and temperature. The catecholamines first condense with formaldehyde to form TIQs, which become further transformed to fluorescent 3,4-dihydroisoquinolines (Figure 3).

**Fig. 3.** Dopamine (I) condenses with formaldehyde to form 6,7-dihydroxy-TIQ (II). In the formaldehyde condensation method for the visualization of dopamine under the fluorescent microscope, II is further transformed to fluorescent tautomers of the corresponding dihydroisoquinoline (III, IV).



Since TIQs are reaction intermediates, it follows that TIQs in tissues can be visualized by the same procedure. Fluorescence microscopy was used to study the uptake and release of these alkaloids by peripheral adrenergic nerves. However, in order to avoid interference from fluorescence of endogenous norepinephrine, the animals were first treated with reserpine or a catecholamine synthesis inhibitor in order to deplete the endogenous norepinephrine.

In studies *in vitro* (21), norepinephrine depleted irides were incubated for thirty minutes at 37 degrees C in isotonic buffer containing norepinephrine, dopamine or 6,7-dihydroxy-TIQ (1-10  $\mu\text{g/ml}$ ). Fluorescence microscopy revealed that 6,7-dihydroxy-TIQ was taken up into the adrenergic plexus of the iris and that it was particularly well accumulated in the varicosities (nerve terminals), even in reserpinized preparations. TIQ accumulation was better than that for dopamine, but about 1/10th that for norepinephrine, as judged by fluorescence microscopy. Uptake was completely blocked by  $10^{-5}\text{M}$  desmethylinipramine. Studies performed by electron microscopy (22) showed that 6,7-dihydroxy-TIQ was stored in the catecholamine-binding vesicles of adrenergic nerve terminals in the iris and pineal gland.

In studies *in vivo* (23), rats were pretreated with alpha-methyl-p-tyrosine methyl ester (500 mg/kg). These animals showed very little evidence of an adrenergic nerve plexus in the iris (Figure 4a) due to depletion of endogenous norepinephrine. Under urethane anesthesia (2 g/kg), 6,7-dihydroxy-TIQ (10 mg/kg) was injected into the femoral vein in order to load the peripheral sympathetic nerve terminals with TIQ. Both cervical sympathetic trunks were then cut, and one trunk was stimulated at parameters that were supra-maximal for a normal animal (6 V amplitude, 2 msec duration, 15 biphasic pulses per sec). After thirty minutes of stimulation, the irides were removed and examined by fluorescence microscopy. The control iris (unstimulated) exhibited a rich adrenergic nerve plexus due to the presence of 6,7-dihydroxy-TIQ (Figure 4b); there were prominent varicosities and the overall appearance was similar to a normal iris filled

with norepinephrine. In contrast, the iris that had been subjected to preganglionic stimulation showed very much less fluorescence intensity, with smaller varicosities and a smoother overall appearance (Figure 4c). The depletion was even greater when desmethylinipramine was used to prevent reuptake of released TIQ.

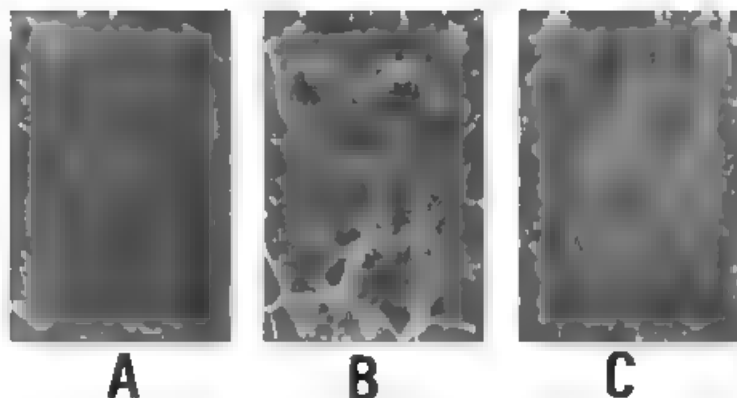


Fig. 4. Fluorescence microscopy was used to study the adrenergic nerve plexus in stretch preparations of irides from norepinephrine-depleted rats (treated with alpha-methyl-p-tyrosine methyl ester, 500 mg/kg). (A) Control iris. (B) After injection of 6,7-dihydroxy-TIQ (10 mg/kg); a rich adrenergic plexus is visible. The plexus is indistinguishable from a normal plexus containing norepinephrine. (C) Depletion of 6,7-dihydroxy-TIQ evoked by stimulation of the sympathetic trunk (see text).

#### AGONIST AND ANTAGONIST ACTIONS OF TIQS

Various investigators have demonstrated the ability of some complex TIQs, such as tetrahydropapaveroline, to activate beta-adrenergic receptors in the bronchioles (24) and in the fat pad (25) or isolated fat cells (18); in these studies, dilation of the bronchioles (24) or release of free fatty acids or glycerol (18,25) was observed. In the studies in which the release of 6,7-dihydroxy-TIQ from nerve terminals was evoked by electrical stimulation of the sympathetic trunk (see above), direct agonist actions of this TIQ on alpha-adrenoreceptors were noted (23). The specific actions that were observed were retraction of the eyelid accompanied by a pronounced protrusion of the eyeball and pupillary dilation (constriction of the iris). Such effects are typical for release of the natural transmitter, norepinephrine. However, norepinephrine was absent due to prior treatment with a catecholamine-synthesis inhibitor, and it was established that 6,7-dihydroxy-TIQ had not been transformed to norepinephrine within the nerve plexus. Because dose-response

relationships (viz, magnitude of electrical stimulation vs. end organ response) were not studied, it is difficult to fully evaluate the efficacy of 6,7-dihydroxy-TIQ as a surrogate neurotransmitter in this system. However, it is noteworthy that responses to electrical stimulation were absent just prior to intravenous injection of the TIQ and that the final response to stimulation was indistinguishable from that in a normal rat. It is not clear from these studies whether 6,7-dihydroxy-TIQ is weaker, stronger or equal to norepinephrine as a transmitter agent for each of the observed parameters of action.

Recent studies have investigated the effects of TIQs on dopamine receptors of the brain. Both agonist actions (stimulation of adenylyl cyclase) (26) and antagonist actions (27) were observed. As a dopamine agonist, 6,7-dihydroxy-TIQ was approximately 1/10th as effective as apomorphine, and 1/100th as effective as dopamine (26). Salsolinol, on the other hand, inhibited the rise in cyclic AMP (27).

In a recent study, the effects of two dopamine-derived TIQs (6,7-dihydroxy-TIQ and salsolinol) on the rat vas deferens were investigated (28). The vas deferens with attached hypogastric nerve was mounted in an organ bath and superfused with modified Krebs' solution at a flow rate of 5 ml/min. The hypogastric nerve was stimulated at a voltage just supra-maximal for each preparation. Stimulation for thirty seconds was repeated every three minutes (one msec duration, twenty pulses per sec). Preparations were first stimulated to record baseline responses. They were then incubated for thirty minutes with TIQ ( $6 \times 10^{-5}M$ ) and subsequently washed with fresh Krebs' solution without TIQ for fifteen minutes. The vas deferens was then retested. In control preparations, responses were unaltered. However, in TIQ-treated preparations, changes in mechanical responses were observed. 6,7-dihydroxy-TIQ suppressed the initial twitch. Additionally, there was a transient potentiation of the second-phase response. Potentiation of the second-phase response could be maintained, however, if 6,7-dihydroxy-TIQ was present in the bathing fluid throughout. S(-)-Salsolinol, on the other hand, markedly suppressed both the twitch and second-phase responses (Figure 5). These actions of 6,7-dihydroxy-TIQ or S(-)-salsolinol could not be produced by incubating the vas deferens with dopamine or with tyramine (a norepinephrine-releasing agent). Therefore, altered responses after exposure to TIQs were not mediated by catecholamines.

The mechanisms for the actions of the TIQs on the vas deferens are not clear. Swedin (29) has noted that the twitch response is inhibited by prostaglandins released during nerve stimulation; perhaps the TIQs increase the release of prostaglandins or augment their effectiveness. On the other hand, diminished responses may imply receptor blockade;

therefore a blocking action on norepinephrine-receptors should be considered.

Fig. 5. The normal response of a rat vas deferens (left) consists of a rapid twitch, followed by a more slowly developing, but more sustained contraction. Prior treatment with  $6 \times 10^{-5}M$  S(-)-salsolinol depressed both the twitch and second phase contraction (right).



#### INHIBITION OF MONOAMINE OXIDASE (MAO) BY TIQS

Yamanaka (30) and Collins et al. (31) showed that two TIQs, salsolinol and tetrahydropapaveroline, were weak inhibitors of MAO in homogenates of rat brain or liver. When serotonin was used as the substrate for MAO,  $K_i$  values in the range 0.14-0.35 mM were reported. However, TIQs sequestered in nerve terminals can be present at a relatively high molar concentration. Therefore, a relatively strong inhibition of intraneuronal MAO by TIQs was a distinct possibility.

More recent studies have tested the actions of TIQs within an intact peripheral adrenergic nerve plexus *in vitro* (32) and *in vivo* (33). In these studies, a reserpinized model was used. It is known that reserpinized nerves lose their capacity to retain  $^3H$ -norepinephrine. Although transport into nerves is apparently normal, the vesicular storage mechanism is blocked and cytoplasmic  $^3H$ -norepinephrine is subjected to degradation by mitochondrial MAO. In normal animals, the binding of NE in vesicles prevents degradation by MAO. Inhibition of MAO can protect cytoplasmic norepinephrine and, thereby, increase the amount of norepinephrine retained within the neuron. However, MAO inhibitors cannot normalize the retention of norepinephrine because access to storage vesicles is blocked in reserpinized animals.

Results of *in vivo* studies (33) are shown in Table 1. In these experiments, mice received injections of reserpine (10 mg/kg). Eighteen hours later, 6,7-dihydroxy-TIQ (10 mg/kg) was injected intravenously. Injection of this dose results in the accumulation of 6,7-dihydroxy-TIQ within nerve terminals of sympathetic nerves (23). Twenty minutes later,  $^3H$ -norepinephrine (100  $\mu Ci/kg$ ) was injected intravenously. Exactly five minutes later, the hearts were removed and analyzed for  $^3H$ -norepinephrine and  $^3H$ -deaminated catechols. Some mice were treated additionally with desmethylinipramine (DMI, 20 mg/kg) at 1.5 hours prior to i.v.  $^3H$ -norepinephrine; DMI blocks the axonal membrane pump for amines and denies entry of  $^3H$ -norepinephrine (as well as 6,7-dihydroxy-TIQ) into adrenergic nerve terminals. The levels of  $^3H$  present specifically in the

adrenergic nerve plexus of the heart was given by the difference between groups of animals with and without pretreatment with DMI.

TABLE 1

*Evaluation of 6,7-Dihydroxy-TIQ as an Inhibitor of Intra-neuronal Monoamine Oxidase Within the Adrenergic Nerve Plexus of the Mouse Heart*

		Radioactivity in Nerve Terminals <sup>a</sup> (DPM per mg Heart $\pm$ SEM)	
		<sup>3</sup> H-NE	<sup>3</sup> H-Deaminated Catechols
Controls	(N = 16)	757 $\pm$ 30	8 $\pm$ 1
Reserpine	(N = 22)	67 $\pm$ 6	91 $\pm$ 6
Reserpine + 6,7-Dihydroxy-TIQ	(N = 23)	283 $\pm$ 59 <sup>b</sup>	68 $\pm$ 4 <sup>b</sup>

a. The radioactivity present in non-neuronal sites in reserpine-treated groups was as follows: <sup>3</sup>H-norepinephrine = 47  $\pm$  4 DPM/mg with and without TIQ, and <sup>3</sup>H-deaminated catechols = 7  $\pm$  3 DPM/mg with, and 6  $\pm$  1 DPM/mg without TIQ.

b.  $p < 0.01$  compared to the reserpine group.

The results of Table 1 show that 6,7-dihydroxy-TIQ raised the accumulation of <sup>3</sup>H-norepinephrine from 8.8% of control to 37.4% of control within the adrenergic nerve plexus. Simultaneously, there was some decrease in levels of <sup>3</sup>H-deaminated catechols. The ratio <sup>3</sup>H-norepinephrine/<sup>3</sup>H-deaminated catechols rose from 0.74 in reserpinized mice to 4.17 in corresponding mice that received i.v. 6,7-dihydroxy-TIQ. These results are comparable to those reported for other MAO inhibitors in similarly designed studies (34,35) and they illustrate the effectiveness of 6,7-dihydroxy-TIQ as an inhibitor of MAO within an adrenergic nerve plexus in vivo. Similar studies, conducted in vitro with isolated mouse atria (32) have shown that salsolinol is also an effective MAO inhibitor.

## CONCLUSIONS

The foregoing experiments illustrate the range of interactions with adrenergic systems that may be anticipated for



TIQs formed endogenously in a spontaneous reaction between acetaldehyde and neuronal catecholamines during intake of alcoholic beverages. TIQs are capable of interfering with mechanisms that regulate the synaptic properties of catecholamines and they, themselves, can be stored and then released from nerves. Upon discharge from nerve terminals TIQs can function either as direct agonists (surrogate transmitters) of adrenergic receptors, or as antagonists to the catecholamines, depending upon the specific receptor area and specific TIQ in question. Because TIQs can be taken back up into nerve terminals and sequestered, and because they are not metabolized by MAO, it is easy to imagine how they might represent a chemical residuum of alcohol persisting into post-intoxication states. Further research is required to determine whether or not the behavioral responses seen during alcohol intoxication or withdrawal are based, in part, on the physiologic actions of small quantities of TIQs that alter the interactions between catecholamine-containing nerve terminals and their receptors.

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## 9. IDENTIFICATION OF ISOQUINOLINE ALKALOIDS DURING ALCOHOL INTOXICATION\*

Michael A. Collins

Department of Biochemistry and Biophysics, Stritch School of  
Medicine, Loyola University of Chicago, Maywood, Illinois.

### INTRODUCTION

Several aromatic amines of neural significance are known to cyclize or condense with aldehydes. The principal products of these reactions are compounds structurally akin to certain phenolic plant alkaloids--1,2,3,4-tetrahydroisoquinolines when catecholamines are the reactants, and 1,2,3,4-tetrahydro- $\beta$ -carbolines when indoleamines are involved. The aldehyde of importance has been acetaldehyde, a metabolic product of ethanol (1,2). Another physiological aldehyde that has received attention is 3,4-dihydroxyphenylacetaldehyde, a normal metabolic intermediate which can cyclize with dopamine to form a 1-benzyl alkaloid, tetrahydropapaveroline (3,4). Also, formaldehyde has received limited consideration in terms of these condensation reactions (5) because it is a methanol metabolite, and is possibly circulating in the alcoholic during chronic ethanol ingestion (6).

Several other chapters in this book and articles elsewhere (1,7) adequately present the background work on catecholamine-derived tetrahydroisoquinolines, and the rationale for suggesting that the alkaloids may be aberrant intraneuronal substances implicated in alcohol dependence or even tolerance. However, in contrast to the rapid expansion of provocative pharmacological findings on tetrahydroisoquinolines which are discussed in the accompanying chapters, research on alkaloid formation appears quite dismal. Indeed, researchers and clinicians must appreciate that it is still not known whether tetrahydroisoquinolines are found in significant amounts in alcoholic drinkers. There are probably a number of reasons why this is unanswered. For example, the

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methods for tetrahydroisoquinoline detection are still expensive, highly specialized and fairly difficult to carry out (with the exception of high pressure liquid chromatography, *vide infra*), and well-controlled human intoxication studies apparently have not been done, or at least have not been reported. At any rate, urinary excretion profiles from such studies conceivably could be unenlightening, even in the face of significant tissue alkaloid synthesis.

In this chapter, recent and past data on the *in vivo* formation of catechol tetrahydroisoquinolines in intoxicated rats will be presented, and its relevance to human alcoholism considered. The data has been obtained mainly from experiments in our laboratory using gas chromatography with electron capture detection (ECD). Several new developments in the methodology of tetrahydroisoquinoline detection, as well as new directions in alkaloid formation, will be stressed.

## METHODS

The experiments discussed here used non-fasted Sprague-Dawley male rats 180-350 g. Drugs (ethanol, pyrogallol, disulfiram) were used as obtained from commercial sources; pargyline was a gift from Dr. S. Speciale. Injection solutions and ethanol gavage solutions were made up in isotonic saline. Disulfiram (Ayerst) was mullied in 1N acetic acid, shaken ten minutes, and titrated to pH 6.75-7.1 with 1N NaOH.

## CATECHOL EXTRACTIONS (8)

Immediately following decapitation, brains were removed and rinsed in cold isotonic saline. Either the entire catecholamine-rich areas (pyrogallol experiments) or the caudates and hypothalami (disulfiram experiments) were dissected out, weighed and homogenized in 1N perchloric acid. Following centrifugation, the supernatants were extracted for catechols with aluminum oxide ( $Al_2O_3$ ). The hydrochloric acid extracts from  $Al_2O_3$  were lyophilized and stored at  $-20^{\circ}C$  prior to derivatization.

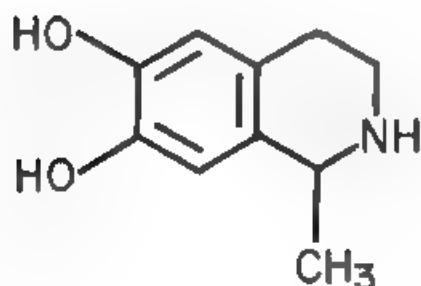
## ANALYSES

For acetaldehyde and ethanol, 200  $\mu$ l blood samples were quantitated on Porapak QS gas chromatographic columns by the head space technique, as described elsewhere (9). Tetrahydroisoquinolines were determined according to the procedure of Bigdeli and Collins (8). Lyophilized acid extracts from tissues were derivatized with heptafluorobutyryl anhydride in acetonitrile, separated on relatively polar (5% OV-17 on 80/100 gas chrom Q) and non-polar (5% SE-54 on 80/100 GCQ) 6 ft. glass

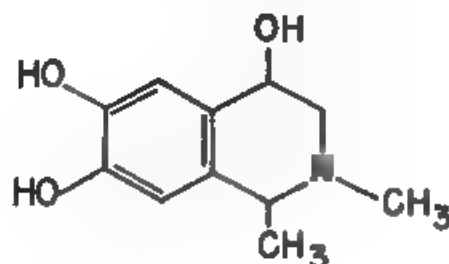
columns, and analyzed by  $^3\text{H}$ - or  $^{63}\text{Ni}$ -ECD (Varian). Derivatized catechols from adrenal glands were separated on 5% GEXF1105 columns. In ethanol experiments using pyrogallol,  $^{14}\text{C}$ -catecholamines were carried through each sample in order to determine recoveries (10). In the disulfiram-ethanol experiments, recoveries were obtained by adding known quantities of salsolinol to selected perchloric acid extracts.

## RESULTS

As discussed in other reports (10,11), treatment of rats with acute or chronic ethanol by liquid diet or gavage failed to produce detectable concentrations of brain salsolinol (SAL), the tetrahydroisoquinoline product of acetaldehyde with dopamine, or of N-methyl-4-hydroxy-salsolinol (MOSAL), adrenal tetrahydroisoquinoline derived from acetaldehyde and epinephrine (Figure 1).



SAL



MOSAL

Figure 1

The combination of pyrogallol (250 mg/kg i.p. sixty minutes before ethanol) and acute ethanol (three i.p. injections, 9 g/kg total, over five hours, with sacrifice two hours later) elevated acetaldehyde blood levels 10-20 fold. Evidence was obtained on two gas chromatographic columns for the formation of SAL in the combined caudate, midbrain, and brain stem areas of pyrogallol/ethanol-treated rats (10). As shown in Table 1, DOPA administration (100 mg/kg i.p.) thirty and 150 minutes after pyrogallol did not significantly change SAL concentrations. However, pargyline pretreatment (100 mg/kg i.p. twenty-four hours and one hour before pyrogallol) caused a dramatic increase in the concentrations of the dopamine-derived tetrahydroisoquinoline (Table 1). Although not consistently assayed, acetaldehyde blood levels were generally similar at sacrifice between these three groups. Gas chromatograms of catechols from controls (saline rather than ethanol) showed no evidence for the presence of SAL.

TABLE 1

*Effect of Pargyline and DOPA on Salsolinol Concentrations  
in the Catecholamine-Rich Brain Regions of  
Pyrogallol/Ethanol-Treated Rats*

	<u>Salsolinol (ng/g + s.d.)</u>
Pyrogallol/Ethanol (5)	18 $\pm$ 9
Pyrogallol/Ethanol + DOPA (6)	29 $\pm$ 15
Pyrogallol/Ethanol + Pargyline (7)	118 $\pm$ 20*

Rats were treated as described in the text. Number of animals in parentheses. \* $p < 0.02$  when compared to pyrogallol/ethanol or pyrogallol/ethanol + DOPA.

Adrenals from pyrogallol/ethanol rats contained a new catechol which chromatographed identically with MOSAL, the suspect epinephrine derivative (Figure 2). DOPA treatment as described above had little effect on adrenal epinephrine and MOSAL concentrations (Table 2). Furthermore, pargyline, contrary to the brain situation, did not significantly elevate either the catecholamine or the adrenal tetrahydroisoquinoline. Ethanol intoxication did reduce adrenal epinephrine levels significantly in pyrogallol-treated rats, and as shown in Table 2, pargyline appeared to block this reduction while DOPA did not.

In similar studies underway with disulfiram and ethanol, disulfiram was intubated daily (25 mg/kg) for one week, and a single dose of 25% ethanol or saline was intubated on the last day. In these studies (Letizia and Collins, in preparation), acetaldehyde blood levels ninety minutes after 3 g/kg and 5 g/kg ethanol were 379 nmoles/ml and 551 nmoles/ml, respectively (Table 3). "Endogenous" acetaldehyde blood levels in disulfiram/saline controls were low but measurable (30-60 nmoles/ml).

Using the dual column technique with  $^3\text{H}$ -ECD, SAL was identified and quantitated in the caudatal and hypothalamic areas of disulfiram/ethanol-treated rats. Concomitant with a 1.5-fold increase in acetaldehyde blood levels, the concentrations of SAL increased by 60-85% (Table 3). There was no indication of salsolinol in the respective brain areas from disulfiram-saline rats. Adrenal MOSAL analyses are in progress.



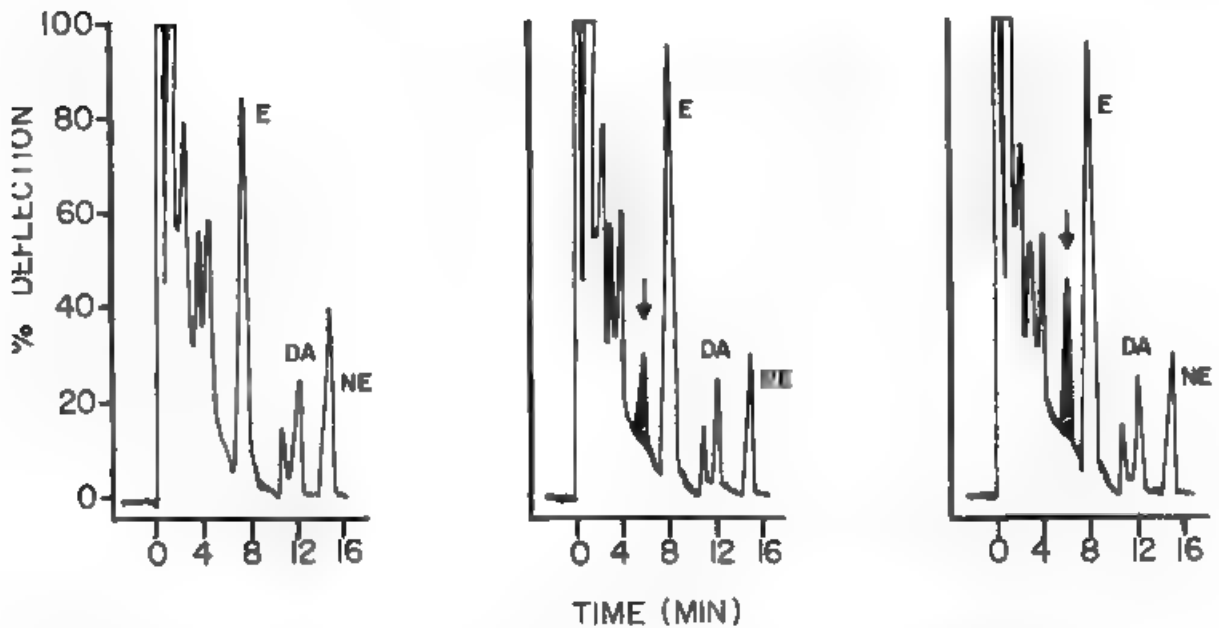


Fig. 2. Three gas chromatograms of the heptafluorobutyryl (HFB)-derivatized catechols in rat adrenals. Chromatogram on the left is the control (pyrogallol-saline) profile, with E (epinephrine), DA (dopamine) and NE (norepinephrine). The center (experimental) chromatogram from rats treated acutely with ethanol following pyrogallol shows the presence of a new catechol (arrow) with a retention time identical to the E-derived tetrahydroisoquinoline, N-methyl-4-hydroxy-SAL (MOSAL). Addition of the HFB-derivatized N-methyl-4-hydroxy-SAL to the experimental sample potentiates this new peak, as shown on the chromatogram on the right.

Conditions: Rats were treated and adrenal catechols were prepared as described in the text. Individual samples derived from single pairs of adrenals were diluted with 10-20 ml. ethyl acetate, and 1-2  $\mu$ l portions were analyzed with  $^{63}\text{Ni}$ -ECD on 5% GEXF1105 gas chromatography columns at 175°C.  $N_2=35$  ml/min, att= $2 \times 10^{-10}$  AFS.

## DISCUSSION

When its oxidation in rats is inhibited by pyrogallol or disulfiram, acetaldehyde derived from administered ethanol cyclizes with brain dopamine (and perhaps other brain catecholamines) to form salsolinol in quantities of 15-50 ng/g tissue; combined pretreatment with pargyline and pyrogallol raises this value to nearly 120 ng/g. As indicated in the preliminary disulfiram/ethanol results (Table 3), salsolinol formation is approximately proportional to the high levels of blood acetaldehyde.

TABLE 2

*Effects of Ethanol, Pargyline and DOPA on Concentrations of N-Methyl-4-Hydroxy-Salsolinol (MOSAL) and Epinephrine in Adrenals of Pyrogallol-Treated Rats*

	Pargyline Adm.	$\mu\text{g/g} \pm \text{s.d.}$	
		MOSAL	EPI
Pyrogallol/Saline (6)	-	undetect.	434 $\pm$ 41
(4)	+	undetect.	390 $\pm$ 95
Pyrogallol/Ethanol (6)	-	3.1 $\pm$ 0.8	224 $\pm$ 39*
(7)	+	4.0 $\pm$ 1.0	327 $\pm$ 55**
Pyrogallol/Ethanol/DOPA (6)	-	3.3 $\pm$ 1.0	216 $\pm$ 64*

Drugs were administered as described in the text. Number of animals in parentheses.

\*  $p < 0.05$  compared to pyrogallol/saline.

\*\*  $p < 0.05$  compared to pyrogallol/ethanol without pargyline.

TABLE 3

*Effect of a Single Ethanol Dose on Salsolinol Concentrations in the Caudate Nucleus and Hypothalamus of Disulfiram-Treated Rats*

Ethanol (p.o.)	Blood Acetaldehyde (nmoles/ml)	Salsolinol (ng/g $\pm$ s.d.)	
		Caudate	Hypothalamus
3 g/kg (5)	379 $\pm$ 58	29.8 $\pm$ 17.7	15.4 $\pm$ 13.9
5 g/kg (8)	551 $\pm$ 97	45.6 $\pm$ 17.2	29.3 $\pm$ 12.4

Rats were treated with disulfiram for one week and ethanol on day 7 as described in the text. Acetaldehyde levels and salsolinol concentrations were assayed according to the procedures in METHODS. Number of rats in parentheses.

As discussed in those chapters by Cohen, Hirst et al. and Blum et al., salsolinol, although not a highly active drug, has definite pharmacological and behavioral effects. In our own studies we have found that peripheral administration of the alkaloid in a dose range of 50-250 mg/kg significantly reduces brain and heart catecholamines (12). This may be the general result of tetrahydroisoquinoline release and inhibition of uptake of catecholamines (1). An alternative is that it reflects, in part, inhibition of catecholamine biosynthesis at the tyrosine hydroxylase locus. Salsolinol and several other catechol tetrahydroisoquinolines, with the notable exception of tetrahydropapaveroline, are reasonably good *in vitro* inhibitors of this regulatory enzyme (12).

Another acetaldehyde-derived alkaloid which appears to be present in our *in vivo* experiments, MOSAL (Figure 1, the derivative of adrenal epinephrine), is of particular physiological interest. This tetrahydroisoquinoline causes long-lasting and nearly complete depletion of guinea pig hypothalamic norepinephrine when given peripherally (in a crude form) at doses of 1-3 mg/kg (13). Such a striking "6-hydroxy-dopamine" effect certainly warrants further study, but facile, practical syntheses of pure 4-hydroxy-tetrahydroisoquinolines are needed (14).

Now considering other analytical approaches, gas chromatography with mass spectrometric (multiple or single ion) detection (GC/MS) has been used to detect low concentrations of dopamine-derived tetrahydroisoquinolines. The first "physiological" demonstration of salsolinol and tetrahydropapaveroline, in fact, was in GC/MS (and ECD) studies of urines from a limited number of Parkinsonian patients undergoing DOPA therapy (15). The following year, researchers in Milan reported GC/MS evidence for tetrahydropapaveroline in whole brain from DOPA-treated rats (16). GC/MS remains the definitive and perhaps the most sensitive technique for the identification and assay of endogenous neuroamines and their metabolites (17).

An important new development in catechol tetrahydroisoquinoline analysis is high performance liquid chromatography with thin-layer electrochemical detection. Riggan and Kissinger have applied this highly sensitive, inexpensive technique [previously developed for analysis of catecholamines (18)] to the detection and quantitation of dopamine-derived tetrahydroisoquinolines (19). They have found SAL, norSAL (6,7-dihydroxy-tetrahydroisoquinoline, from dopamine and formaldehyde) and tetrahydropapaveroline excretion by rats treated only with 100 mg/kg DOPA (19). High performance liquid chromatography provides a relatively simple alternative to gas chromatography with ECD, and if used in conjunction with the latter technique, would substantiate the identities

and amounts of amine-derived alkaloids.

If radioactive precursors are employed, gas chromatography with radioactivity detection (gas radiochromatography) can be a valuable identification tool. Frequently used in  $^{14}\text{C}$ -bio-synthetic studies, gas radiochromatography has been used recently to identify the dopamine-formaldehyde product, norSAL (6,7-dihydroxy-tetrahydroisoquinoline), during incubations of the catecholamine and methyl-tetrahydro[5- $^{14}\text{C}$ ] folic acid with a "formaldehyde-forming" enzyme preparation from rat brain (20). The principal enzyme is believed to be 5,10-methylene-tetrahydrofolate reductase (21,22) rather than a biogenic amine N-methylating enzyme. Tetrahydroisoquinolines (23) or tetrahydro- $\beta$ -carbolines (24) were found to be the apparent *in vitro* products, and gas radiochromatography helps to confirm this. The general technique could be applied to *in vivo* studies with ethanol and  $^{14}\text{C}$ -precursors, providing the necessary cold carrier alkaloid products are available to aid in the collection of the gas chromatographic peaks.

The results of our gas chromatography/ECD experiments on tetrahydroisoquinoline formation in high acetaldehyde situations provoke questions about the actual feasibility of extensive aldehyde cyclization *in vivo*. Since the quantities of SAL and MOSAL are admittedly low in the face of high blood acetaldehyde, are there tissue mechanisms which preclude the cyclization pathway? It appears to be so. Possibilities include low acetaldehyde levels within tissues, rapid acetaldehyde oxidation, unavailability of the major proportions of catecholamines, and thiol group interactions with the aldehyde. The first possibility, perhaps a factor for the brain--Sipple finds brain acetaldehyde to be ca. 40-60 nmoles/g at 300 nmoles/ml blood levels (25), and Tabakoff et al. report that acetaldehyde levels in mouse brain to be 10% of the blood levels (26)--is regarded as unlikely because of the limited tetrahydroisoquinoline formation in the adrenals, which should have no great acetaldehyde barrier. Acetaldehyde oxidation may be an important factor, although aldehyde dehydrogenase inhibitors were administered in these experiments. More likely is protection of stored or vesicular catecholamines from the cyclization reactions [a factor which may be overcome by pargyline treatment in our studies, or by DOPA ingestion (16)]. We are also considering the fourth (but not final) possibility that *in vivo* acetaldehyde is highly and reversibly bound to tissue thiol groups and thus is relatively unavailable for interaction with biogenic amines.

Our failure to detect SAL or MOSAL in rat tissues following ethanol intoxication in the absence of enzyme inhibitors has been confirmed in part by Riggin and Kissinger (19). Furthermore, Rahwan and O'Neill, using gas chromatography with ECD, were unable to detect SAL in whole brains of mice

following six days exposure to ethanol vapor (27). These authors conclude that SAL has no role in ethanol effects in mice.

Presently, although others may differ (28,29), we do not interpret these data to mean that acetaldehyde interactions with nucleophilic functions in general and with catecholamines, specifically, are necessarily insignificant molecular processes in human alcoholism. Because of possible differences between rodents and humans in the physiology of alcohol dependence, and perhaps in such biochemical aspects as acetaldehyde tissue uptake, levels and turnover, biogenic amine availability and tetrahydroisoquinoline disposition, results from intoxicated rodents can not be extrapolated readily. Biochemical studies to date in rodents may even be overlooking more important acetaldehyde/nucleophile interactions, e.g., with thiol-containing (endogenous) substrates (30) or membrane proteins. Clearly, further animal work and rigorous, direct human exploratory studies using several of the sensitive techniques reviewed here are needed before alkaloid formation can be discounted.

#### SUMMARY

With dual column gas chromatography and sensitive electron capture detection (ECD), catechol tetrahydroisoquinoline derivatives of endogenous catecholamines can be identified and assayed in neural tissues of rats treated with ethanol and inhibitors of acetaldehyde oxidation. Thus, salsolinol (SAL) and N-methyl-4-hydroxy-salsolinol (MOSAL), alkaloidal products of acetaldehyde condensation with dopamine and epinephrine, respectively, are present in low levels in rat brain (SAL) and adrenals (MOSAL) following pyrogallol and ethanol. Disulfiram pretreatment followed by acute intoxication also results in amounts of caudatal SAL which are proportional to the elevations in blood acetaldehyde as ethanol dosage is increased. Attempts to demonstrate tetrahydroisoquinoline biosynthesis in intoxicated rats with low acetaldehyde blood levels (no inhibitors) have been unsuccessful to date with gas chromatography and ECD. Other methods--gas chromatography with mass spectrometric or radiochemical detection, or the promising new high performance liquid chromatography with electrochemical detection--may have the sensitivity required. Other species would offer more promise than rodents, and, of course, rigorous examination of body fluids from human alcoholics during conditions of controlled diet and subsequent intoxication may provide answers to the controversy over the importance of acetaldehyde and tetrahydroisoquinolines in alcoholism.

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## 10. PHARMACOLOGY OF ISOQUINOLINE ALKALOIDS AND ETHANOL INTERACTIONS\*

Maurice Hirst, Murray G. Hamilton and Alice M. Marshall

The University of Western Ontario, Department of Pharmacology,  
London, Ontario, Canada.

### INTRODUCTION

Very few of the more than sixty naturally-occurring isoquinoline alkaloids have been screened for pharmacological activity, although certain members of this class have known hallucinogenic and cardiovascular activity. Recently the suggestion that biosynthetic catecholamine-derived tetrahydroisoquinolines are involved in the etiology of alcoholism has stimulated research into their activity. Both salsolinol and its desmethyl analogue, 6,7-dihydroxytetrahydroisoquinoline, have been shown to exacerbate ethanol withdrawal convulsions in mice. However, the action of such isoquinolines in altering the behavioural expression of acute ethanol intoxication has not been reported. The results presented here demonstrate the ability of salsolinol and 3-carboxysalsolinol and their non-cyclised amine precursors, dopamine and L-DOPA respectively, to increase the duration of ethanol-induced narcosis in mice. The carboxylated isoquinoline was the most potent of the four compounds tested. Pretreatment with the alcohol dehydrogenase inhibitor pyrazole significantly lengthened the narcosis produced by ethanol and by both of the isoquinoline-ethanol combinations. Disulfiram, an inhibitor of both aldehyde dehydrogenase and dopamine-beta-hydroxylase, led to an increased sleeping time after either ethanol or L-DOPA-ethanol treatments, but had no effect on the isoquinoline ethanol narcosis. When carbidopa, an inhibitor of peripheral L-amino-acid decarboxylase, was administered, a significant increase in duration of narcosis following either L-DOPA or 3-carboxysalsolinol with ethanol was obtained. The results show that simple isoquinolines such as salsolinol and 3-carboxysalsolinol potentiate the narcotic effect of a single

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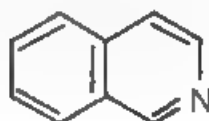
injection of ethanol and suggest that *in vivo* formation of such compounds may play a significant role in both acute and chronic alcoholism.

#### PHARMACOLOGICAL ACTIONS OF NATURAL AND SYNTHETIC ISOQUINOLINES

Isoquinoline alkaloids occur widely in nature. They are to be found in many families of plants, including species of Anonaceae, Berberidaceae, Cactaceae, Chenopodiaceae, Combretaceae, Fumaraceae, Hernandiaceae, Lauraceae, Leguminosae, Magnoliaceae, Menispermaceae, Monimiaceae, Nymphaeaceae, Papaveraceae, Ranunculaceae, Rhamnaceae and Rutaceae (1).

On structural grounds this group of natural products can be divided into two major categories: the simple isoquinolines which possess only one aromatic nucleus and the benzylisoquinolines which contain two such nuclei (Figure 1). Both

Simple Isoquinoline



Benzylisoquinoline

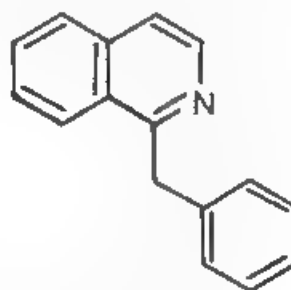


Figure 1

categories can be expanded to include phenol-ether linked dimers and trimers. Many other groups of alkaloids are structurally related to the benzylisoquinolines. These include the protoberberines, protopines, pavines, aporphines and the phthalide-isoquinolines (1). In some plants the isoquinolines are present in combination with biosynthetic precursor phenethylamines (2,3); in others, isoquinolines are primogenitors of more complex alkaloids (1).

While the isoquinolines are the most numerous of all the groups of alkaloids, very few of the more than sixty natural isoquinolines have been screened for pharmacological activity. Sporadic investigations into their actions have taken place for more than one hundred years (4), with results accruing from signs and symptoms during toxicity and effects on respiration, the cardiovascular system and visceral smooth muscle.

Trioxytetrahydroisoquinolines present in the peyote cactus *Anhalonium lewinii* Britton and Rose [*Lophophara williamsii* (Lemaire) Coulter] were among the isoquinolines

tested by Heffter in 1898 (5). These substances are central nervous system stimulants. The most potent base, lophophorine, produced hyperexcitability and accelerated respiration in doses of 7 mg/kg in rabbits. An increase in blood pressure was elicited by 2.5 mg/kg with large doses causing a decrease. There was no effect on the heart (5). Another isoquinoline, anhalonidine, did not produce significant symptoms in mammals (5). A more recent study has confirmed these results for Brossi et al. (6) have demonstrated that anhalamine, anhalonidine and pellotine have little activity as sedatives, anti-convulsants, or tranquilizers.

Mescaline is the major alkaloid present in the peyote cactus. The ability of this phenethylamine to cause hallucinations is, without doubt, the reason for the use of sacramental mescal buttons in the religious ceremonies of the Native American Church (7). Co-existing tetrahydroisoquinolines may not have hallucinogenic properties (6) and are not considered to contribute to the psychotogenicity of peyote (8).

The suaharo cactus, *Carnegiea gigantea* (Engelmann) generates two isoquinolines, carnegine and gigantine. Carnegine has pharmacological properties that are similar to the isoquinolines in peyote, provoking convulsions in mammals (9). The more recently discovered alkaloid gigantine (10) is of particular interest. This tetrahydroisoquinoline is an isomer of pellotine, differing only in the transposition of a phenolic hydroxy group (11). Gigantine is considered to be an hallucinogen in squirrel monkeys and cats (10).

Species of *Chenopodiaceae* elaborate the 6,7-dioxytetrahydroisoquinolines salsoline and salsolidine. Both alkaloids are vasodilators and respiratory stimulants (12). These effects may be mediated by sympatholytic actions for the alkaloids are reported to be antagonists to pressor responses induced by adrenaline (12). Salsoline has antihistaminic activity (13), a mild antidiuretic effect mediated, in part, by an action of the compound on the hypothalamic-hypophyseal system (14) and the unusual property of increasing the rate of coagulation of whole or oxalated blood (15). It is one of the few isoquinolines of therapeutic value, being used in the U.S.S.R. in the therapy of hypertension and cerebral angiospasm (16).

Cotarnine, an isoquinoline associated with, and possibly derived from, the phthalide-isoquinoline narcotine has sympatholytic (17,18) and analgesic effects (19). It, and the related isoquinolines hydrastinine and hydrastine are less effective inhibitors of the cough reflex than narcotine (20,21).

Still fewer naturally occurring benzylisoquinolines have been tested pharmacologically. Laudanosine, given intravenously, elicits convulsions in dogs that are prevented by

intraspinal anesthesia (22). Its effects on peripheral structures are qualitatively similar to papaverine (23,24). This latter alkaloid is well-established as a smooth muscle relaxant (25). As recent studies have shown an accumulation of cAMP in smooth muscle treated with papaverine, it is probable that the general relaxant properties reflect an ability to inhibit phosphodiesterase (26). The spasmolytic activity shown by papaverine can be extended to many synthetic analogues (27), some of which are of therapeutic utility (25, 27).

Papaverine is a weak analgesic and, unlike laudanum, depresses the central nervous system (28). The disparity in central activity has been observed with other benzylisoquinolines which differ in containing aromatic or reduced isoquinoline nuclei (29).

The pharmacological properties of a large number of synthetic simple isoquinolines have been investigated by Hjort et al. (30-33). Like their natural alkaloid analogues these substances have central nervous system stimulant or depressant qualities. Variant effects are also produced on the cardiovascular system and on isolated smooth muscle. In the main, compounds that contained a catecholamine moiety were found to be predominantly pressor in action; there was evidence of tachyphylaxis, and there was a potentiation of adrenaline. The analogous ethoxy and methoxy derivatives were more toxic, elicited either biphasic depressor-pressor, or solely depressor effects and frequently inhibited the cardiovascular response to adrenaline (32).

#### ISOQUINOLINE ALKALOIDS AND ETHANOL

The isoquinolines formed in plants are biosynthesised from precursor phenethylamines. These probably condense with keto-acids or aldehydes to yield the cyclised products (1). Analogous reactions occur, *in vitro*, under pseudophysiological conditions (34).

Ethanol is oxidatively metabolised, *in vitro*, to acetaldehyde and acetate. The primary enzymes involved are alcohol and aldehyde dehydrogenase (35). These require as co-factor, nicotinic adenine dinucleotide, NAD. During oxidation the co-factor is reduced to its protonated form, NADH. The process of ethanol oxidation can alter the biochemic redox potential of the body (36), which can lead to aberrant metabolism of endogenous monoamines. It has been shown that the excretion of acidic metabolites of 5-hydroxytryptamine, noradrenaline and adrenaline are depressed with concomitant increases in reduced catabolites, following ethanol consumption (37,38). A significant factor in the alteration of normal monoamine metabolism is considered to be competitive

inhibition, by acetaldehyde of aldehyde dehydrogenase (39,40). This effect prolongs the half life of intermediate aldehyde metabolites (40). Several investigators have considered the possibility that such reactive intermediates could condense with endogenous neuroamines to generate isoquinolines (41,43).

In 1970, Davis *et al.* and Cohen and Collins reported the *in vitro* formation of tetrahydroisoquinolines after exposure of biological systems to acetaldehyde or ethanol (42-45). The generated isoquinolines are salsolinol derivatives of catecholamines and acetaldehyde, and tetrahydropapaveroline formed by the condensation of dopamine with its own intermediate aldehyde, 3,4-dihydroxyphenylacetaldehyde (Figure 2). The

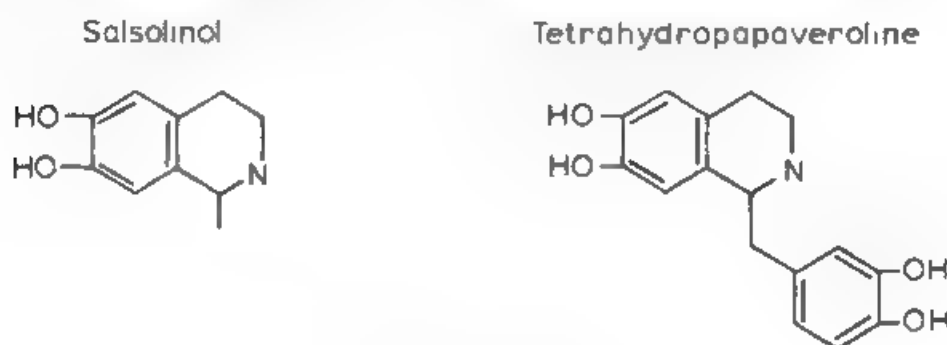


Figure 2

benzylisoquinoline, tetrahydropapaveroline, can be further metabolized by mammalian systems to isoquinoline-based protoberberines (46,47). Others have subsequently identified salsolinol and tetrahydropapaveroline *in vivo* following ethanol administration (48-50). Similar reactions occur with biogenic tryptamines, generating triple ring structures called harmans or  $\beta$ -carbolines. Dajani and Saheb have identified  $\beta$ -carbolines in urine after ethanol administration to rats (51).

On kinetic grounds the condensation of acetaldehyde with biogenic phenethylamines is most likely to occur with dopamine, followed by L-DOPA and then noradrenaline (52).

A further series of endogenous simple tetrahydroisoquinolines may form from condensations of catecholamines with a 1-carbon unit, the prerequisite carbon atom being transferred by way of 5-methyltetrahydrofolate. Vandenheuvel *et al.* have shown that 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline is formed when dopamine is incubated with the 1-carbon unit donor (53). The additional carbon atom required for elaboration of the protoberberine skeleton from tetrahydropapaveroline may also involve 5-methyltetrahydrofolate (46,47). The intermediacy of N-methyl groups in this cyclisation process is known to occur in plants (54-56).

Recent reviews of the pharmacological actions of the

TABLE 1

PHARMACOLOGICAL ACTIVITY OF ALCOHOL RELATED TETRAHYDROISOQUINOLINES

COMPOUND	SMOOTH MUSCLE	CENTRAL NERVOUS SYSTEM
6, 7 dihydroxy- 1, 2, 3, 4 tetrahydroisoquinoline	$\alpha$ sympathomimetic (30, 61, 62) $\beta$ sympathomimetic (30, 61)	hypothermia (intraventricular infusions) (81) exacerbates ethanol withdrawal convulsions (82) produces convulsions (intracerebral infusions) (82) convulsions blocked by naloxone (83)
1 methyl-6, 7 dihydroxy 1, 2, 3, 4-tetrahydro-isoquinoline (salsolinol)	Dopamine agonist (63) Dopamine antagonist (64) $\beta$ sympathomimetic (65) $\alpha$ antagonist (62, 66, 67) 5 hydroxytryptamine antagonist (67, 68) partial agonist at opiate receptors (69) antagonist of vasopressin and oxytocin (66)	biphasic hypo-hyperthermia (intraventricular infusions) (84) potentiates ethanol narcosis (85) depletes cerebral brain $Ca^{++}$ levels (86)
4, 6, 7 trihydroxy- 1, 2, 3, 4 tetrahydro-isoquinolines	$\alpha$ -agonist (70) $\beta$ agonist (70) $\beta$ antagonist (70)	
1-(3', 4' dihydroxy-benzyl)-6, 7-dihydroxy- 1, 2, 3, 4 tetrahydro-isoquinoline (tetrahydropapaveroline)	$\beta$ agonist (71-80)	exacerbates Parkinsonian tremors (72) tricyclic anti-depressant like activity (72)
Tetrahydropapaverolines		tranquilization (88-91) sedation (92-95) analgesia (76-97)

TABLE 1 (continued)  
PHARMACOLOGICAL ACTIVITY OF ALCOHOL RELATED TETRAHYDROISOQUINOLINES

BIOCHEMICAL PHARMACOLOGY			
Effects on Monoaminergic Enzymes		Effects on Control Mechanisms	Effects on Adenylate Cyclases
MAO	COMT		Dopamine-type $\beta$ type
inhibitor (98,100)	substrate (102)	inhibits noradrenaline, dopamine, 5-hydroxytryptamine uptake (104,107) noradrenaline releaser (107)	agonist (108)
inhibitor (99,100,101)	substrate (100)	inhibits noradrenaline dopamine, 5-hydroxytryptamine uptake (46,106)	inhibitor (64) no activity (64)
	inhibitor (100)	releases noradrenaline dopamine, 5-hydroxytryptamine (46,106)	no agonist activity (108)
inhibitor (100)	substrate (103)	depletes regional noradrenaline (heart, aorta hypothalamus) (70)	inhibitor (64) agonist (64)
	substrate (100) inhibitor (100)	inhibits noradrenaline uptake (46)	
		releases noradrenaline (46) inhibits dopamine uptake (46)	

mammalian-based isoquinolines have been published (57-60). Table 1 summarizes the smooth-muscle, central nervous system and biochemical pharmacological activities possessed by these compounds.

The formation of "abnormal" metabolites of central neuroamines following ethanol administrations is of biochemical interest. If such substances interact with the consumed ethanol to alter the acute or chronic effects of the alcohol, then they are of pharmacological importance and have to be regarded as more than metabolic curiosities (109).

Interactions of ethanol with the alcohols derived by reductive catabolism of dopamine, tryptamine and 5-hydroxytryptamine have been demonstrated (110,111). Feldstein et al. in 1970 (110), found tryptophol and 5-hydroxytryptophol to be central nervous system depressants, inducing sleep in mice. When co-administered with ethanol there was a potentiation of ethanol-induced sleeping time. In related studies, Blum et al. (111), showed that tryptophol and 3,4-dihydroxyphenylethanol acted synergistically with ethanol, prolonging ethanol sleep-time. This interaction did not seem to be related to an alteration of ethanol metabolism. In a parallel series of experiments, the neuroamines dopamine and 5-hydroxytryptamine themselves, in much lower doses, protracted ethanol-induced depression, confirming the earlier results of Rosenfeld (112).

These investigations have revealed that one series of abnormal metabolites can alter an acute action of ethanol. However, the neuroamine-derived alcohols cannot be considered to be inert biochemically. It is not improbable that the administered biogenic alcohols could be oxidised by an alcohol dehydrogenase to reactive aldehydes. These products could then react with endogenous neuroamines. Similarly, the neuroamines, co-administered with ethanol, could yield isoquinoline or  $\beta$ -carboline condensates, by reacting with acetaldehyde.

#### EFFECTS OF ISOQUINOLINE ALKALOIDS ON ETHANOL-INDUCED NARCOSIS

A series of experiments were performed to see if ethanol sleeping time could be influenced by isoquinoline bases. Ethanol was given alone or in combination with salsolinol or the related compound, 3-carboxysalsolinol. Comparative experiments were conducted with dopamine and L-DOPA. Some studies were repeated after pretreatment of animals with drugs known to alter the metabolic disposition of ethanol or suppress the peripheral decarboxylation of the amino-acid compounds.

#### MATERIALS AND METHODS

Swiss-Webster albino male mice (24-32 g) were used in



the experiments. The sleeping time protocol employed was that developed by Kakihana et al. (113). All experiments were conducted during the late morning. After drug administration the time at which each mouse lost the righting-reflex was noted. The narcosis duration endpoint was considered to occur when the subject has regained the reflex twice within a thirty second interval. Artifactual rightings, caused by intermittent leg jerks during the narcosis, were negated by this method. A value of 150 minutes was arbitrarily recorded if the subject was still without the righting reflex after that time.

Each of the four test compounds, namely L-DOPA (60 and 1000  $\mu\text{M/kg}$ ), dopamine (60  $\mu\text{M/kg}$ ), salsolinol (60, 460 and 920  $\mu\text{M/kg}$ ) and 3-carboxysalsolinol (7.5, 15, 30 and 60  $\mu\text{M/kg}$ ) was prepared in saline. The test compounds were given (in a 5  $\mu\text{l/g}$  body weight volume) immediately prior to ethanol administration. Delivery of ethanol was made in the form of a 25% (v/v) solution in saline, in a dose of 4 g/kg. The route of administration for all drugs was intraperitoneal, unless otherwise stated. Pretreatments with enzyme inhibitors included administration of pyrazole (34 mg/kg) thirty minutes prior to the above drugs; carbidopa (25 mg/kg) given orally in a gum acacia suspension one hour before other substances; and disulfiram (75 mg/kg) given intraperitoneally in a suspension in acacia twenty-four hours before other treatments. Each pretreatment series had a saline-treated control. The means and standard errors of the means for experimentally determined sleep-times were calculated. Student's t-tests were used to determine levels of significance.

The two isoquinolines used in these experiments were synthesised in our laboratory. The synthesis developed by Buck (32) was used as a basis for salsolinol preparations (66). Its carboxylated analogue, 3-carboxysalsolinol was synthesised by the method of Brossi et al. (114). L-DOPA and dopamine were supplied by Sigma Chemical Co., St. Louis, Missouri; carbidopa was generously donated by Merck and Co., West Point, Pennsylvania.

The four test compounds were injected either alone or in combination with ethanol. A reference sleeping time was obtained upon the administration of saline (5  $\mu\text{l/g}$  body weight) with ethanol. Several combination experiments were repeated, incorporating the pretreatments indicated above. Tests were conducted on groups containing at least ten mice per group.

## RESULTS

The experimental values for durations of sleep-time with the various treatments and combinations of treatments are

TABLE 2

POTENTIATION OF ETHANOL-INDUCED NARCOSIS  
BY AMINES AND ISOQUINOLINES  
AND THE EFFECT OF PRETREATMENTS  
WITH ENZYME INHIBITORS

Pretreatment	Saline (min)	Pyrazole (34mg/Kg) <sup>a</sup> (min)	Disulfiram <sup>b</sup> (75mg/Kg) (min.)	Carbidopa (25mg/Kg) (min)
<u>Treatment(s)</u>				
Ethanol (4g/Kg)	64.3 ± 3.7	96.4 ± 11.5**	80.2 ± 8.6*	54.2 ± 4.5
L DOPA + Ethanol				
60 µM/Kg	75.8 ± 6.2	—	104.0 ± 10.0**	64.5 ± 11.9
250 µM/Kg	52.3 ± 10.8	—	—	78.5 ± 7.3**
1000 µM/Kg	93.0 ± 10.4*	73.0 ± 12.8	—	121.9 ± 8.0*
Dopamine & Ethanol				
60 µM/Kg	81.4 ± 8.2*	91.6 ± 11.1	88.8 ± 8.9	
Salsolinol + Ethanol				
60 µM/Kg	66.8 ± 6.6	96.2 ± 6.3**	78.3 ± 5.7	
460 µM/Kg	82.9 ± 7.2*	—	—	
920 µM/Kg	97.3 ± 9.2*	—	—	
3 Carboxysalsolinol + Ethanol				
7.5 µM/Kg	64.5 ± 9.1	—	—	119.4 ± 9.8***
15 µM/Kg	82.4 ± 8.7*	—	—	
30 µM/Kg	78.7 ± 5.8*	—	—	
60 µM/Kg	109.3 ± 6.3***	133.5 ± 8.3***	100.1 ± 13.1	95.9 ± 11.9

The levels of significance are interpreted as follows

\*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ . The saline pretreatment values have been compared with respect to the value obtained with ethanol alone (64.3 ± 3.7 min). Those values in the remaining columns have been compared with the saline pretreatment values

a This dose of pyrazole was chosen to influence ethanol metabolism yet provide experimental time so that alterations in ethanol-drug-induced narcosis could be determined. At a 68mg/Kg dose the sleeping time after ethanol was 117.5 ± 16.8 min.

b A progressive potentiation of ethanol-induced sleep was obtained with increasing doses of disulfiram. With 150 mg/Kg the ethanol sleeping time was 88.3 ± 9.0 min, with 300 mg/Kg the sleeping time was 101.9 ± min

shown in Table 2. Times of onset of sleep for experimental and ethanol-saline treated control mice were not significantly different. Dopamine, L-DOPA, salsolinol and 3-carboxysalsolinol, alone and after inhibitory drug pretreatments, were without obvious effect in the absence of ethanol. Several treatment combinations protracted ethanol induced sleeping time. Significant prolongations ( $p < 0.05$ ) occurred when ethanol was given at the same time as 1 mM L-DOPA, 60 µM dopamine, 460 µM salsolinol, or 15 µM 3-carboxysalsolinol. Higher doses of the isoquinoline compounds further increased the sleeping times.

Pretreatments with agents that alter the metabolism of ethanol increased the central depressant effect. Both suppression of alcohol dehydrogenase by pyrazole (115-117) and aldehyde dehydrogenase by disulfiram (118) significantly prolonged ethanol narcosis. The pyrazole-ethanol sleeping-time was not altered by the addition of 1 mM L-DOPA, 60  $\mu$ M dopamine, or 60  $\mu$ M salsolinol, although it was elevated by 60  $\mu$ M of 3-carboxysalsolinol.

As mentioned, disulfiram pretreatment increased ethanol sleep-time. This was further increased when 60  $\mu$ M L-DOPA was given at the same time as ethanol. Co-administrations with doses of dopamine, salsolinol or 3-carboxysalsolinol had no influence on the basal disulfiram-ethanol sleep-time.

In contrast, inhibition of L-amino acid decarboxylase by carbidopa did not alter ethanol-induced narcosis. Sleep-times were increased in carbidopa-pretreated animals receiving injections of ethanol and 250  $\mu$ M or 1 mM of L-DOPA or 7.5  $\mu$ M of 3-carboxysalsolinol.

## DISCUSSION

### *POSSIBLE MECHANISMS OF ISOQUINOLINE ETHANOL-INTERACTIONS*

The isoquinoline alkaloids found in plants show many pharmacological properties in common with those derived following alcohol administrations to mammals. While they differ appreciably, in that the plant alkaloids show more variable levels of oxidation and the majority of oxygen substituents exist as methoxy or methylenedioxy moieties, both groups produce effects on smooth muscle and in the central nervous system. The non-mammalian alkaloids have not been exposed to the definitive levels of biological investigation that have centered on the ethanol-related isoquinolines (Table 1). Nonetheless, many of the effects exerted by these compounds may be mediated through the same direct or indirect mechanisms demonstrated for those of mammalian origin.

The results of experiments incorporating dopamine and L-DOPA-derived isoquinolines illustrate that these compounds can interact, acutely, with ethanol. This phenomenon is supported by the results contained in Table 2. Salsolinol, and its more potent amino-acid analogue, demonstrated the capacity to prolong the ethanol-induced sleep-time. In confirmation of the results of others (112,119) this was also shown for dopamine and L-DOPA.

Preservation of the administered ethanol, by inhibiting alcohol dehydrogenase with pyrazole, a procedure employed by Goldstein and Pal (120), increased the ethanol sleep-time. A further prolongation was observed in animals treated additionally with 3-carboxysalsolinol, but not with the other

tested compounds in the doses used. As the conversion of ethanol to acetaldehyde should be suppressed by the pyrazole pretreatment, there should be a corresponding decline in available acetaldehyde. Reduced quantities of this intermediate could attenuate the formation of abnormal metabolites from dopamine and L-DOPA (biogenic alcohols or derived isoquinolines) and, therefore, not allow synergistic interactions with ethanol to occur. Salsolinol may not have prolonged sleeping time after this treatment because of inadequate dosage. The 60  $\mu$ M quantity of this compound with ethanol did not prolong sleeping time compared to the ethanol-treated control.

Disulfiram-treated animals slept longer after exposure to ethanol than control mice. This drug disrupts the facile conversion of acetaldehyde to acetate (118) as well as suppressing the conversion of dopamine to noradrenaline (121,122). The increased sleeping time observed may occur from reaction between elevated levels of acetaldehyde with endogenous neurotransmitter substances and further interactions of these with ethanol. Of significance was the observation that L-DOPA potentiated ethanol-induced sleep-time, in disulfiram-pretreated mice, in a dose that had no significant effect on non-pretreated animals. It is possible that the higher level of the aldehyde was sufficient to alter the metabolism of the amino acid, to yield abnormal metabolites from dopamine which interacted further with the ethanol.

The isoquinolines, salsolinol and 3-carboxysalsolinol, did not increase the sleeping time of ethanol-treated, disulfiram-pretreated mice. This would suggest that the isoquinolines are synergistic with ethanol rather than acetaldehyde. Disulfiram did not prolong the sleeping time associated with the co-administration of dopamine and ethanol. This could reflect the generation of an optimal quantity of the isoquinoline at lower acetaldehyde levels than ones induced by disulfiram. Acetaldehyde reacts more than twice as efficiently with dopamine than it does with L-DOPA to produce isoquinolines (52). Other doses of dopamine, ethanol and disulfiram are being examined to investigate this possibility.

Pretreatments with carbidopa increased sleeping times after ethanol and the amino-acids. The prevention of peripheral decarboxylation by this agent (123) implies that greater quantities of the administered L-DOPA or 3-carboxysalsolinol from biogenetic or synthetic sources, gain access to the central nervous system for interaction with ethanol. It is not known at this time if 3-carboxysalsolinol is a substrate for brain L-amino-acid decarboxylase and can be made into salsolinol *in situ*, but it is pertinent that inhibition of the peripheral and central enzyme by the less specific agent RO 4-4602 prevented L-DOPA from potentiating

ethanol-induced sleep (124).

Isoquinolines can interact with other phases of ethanol intoxication. Amit and Sutherland (125) cite studies by Duby and Amit that show that ethanol-preferring rats, which are pretreated with  $\alpha$ -methyl-p-tyrosine and then infused with an isoquinoline into the lateral hypothalamus, drink less ethanol than controls. This discovery is similar to results obtained by Geller et al. (126) who found that a  $\beta$ -carboline, administered intraperitoneally, reduced voluntary alcohol consumption in rats.

Further interactions of isoquinolines and ethanol are reported by Blum et al. (82). An intracerebral injection of 50  $\mu$ g of 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline increased the severity of withdrawal seizures in ethanol-dependent mice. Lower doses had no effect or reduced the severity of this withdrawal response.

In summary, isoquinolines, isolated from plants or produced in mammals after treatments that incorporate ethanol, are capable of eliciting diverse pharmacological effects. Some isoquinolines can increase the degree of central depression induced by ethanol and influence other aspects of ethanol intoxication. In view of the multiple mechanisms of action associated with these compounds it is not possible to reconcile the interactions of the isoquinolines with ethanol in a discrete manner. A definitive role for cyclised neuroamines, in alcoholism, awaits future clarification. However, the present state of knowledge suggests that these substances may be of critical importance in establishing a biochemical basis for this disorder.

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## 11. OPIATE-ETHANOL INTERACTION STUDIES

Andrew K.S. Ho, Raymond C.A. Chen and J. Michael Morrison\*

Peoria School of Medicine, University of Illinois, Peoria, Illinois, and National Institute on Drug Abuse, Rockville, Maryland.

### INTRODUCTION

Widespread use of alcohol among addicts has been reported by many investigators. Jackson and Richman (1) noted that many narcotic addicts would use alcohol when drugs such as heroin, morphine or cocaine were not available. In New York City during the period from 1950 to 1961 (2) one-tenth of the deaths among narcotic addicts were attributed to the combined use of narcotics and alcohol. Baden (3) reported that more than one-fifth of the heroin addicts who died in New York City had evidence of alcohol abuse. Addicts participating in methadone maintenance programs reported concomitant heavy use of alcohol (4,5). Heroin addicts maintained on prolonged methadone treatment and secondarily addicted to alcohol, develop a ten-fold increase in mortality over those who were on methadone maintenance alone (6). Similar increases in lethality were reported in animals treated with both narcotics and alcohol. In mice pretreated with alcohol, morphine administration markedly increased lethality (7). The depressant effects of alcohol are markedly potentiated by morphine (8). Sinclair *et al.* (9) observed that the selection of alcohol in Sprague-Dawley rats was suppressed after a single injection of morphine (60 mg/kg). In our laboratory, we have used both rats and mice to study the interaction between narcotics and alcohol.

To further clarify opiate-ethanol interactions, and in particular, to test whether previous exposure to either opiate agonists or antagonist (acute, chronic and withdrawal following chronic administration) affect alcohol selection, the following experiments were performed. Preliminary results of these

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studies have been reported elsewhere (10,11).

## MATERIALS AND METHODS

### ANIMALS

The following strains of rats and mice were used: (a) adult male Sprague-Dawley rats weighing 180-200 g; (b) adult male Long-Evans Hooded rats weighing 180-220 g; (c) adult male C57BL/J6 mice weighing 20-25 g; and (d) neonatal Sprague-Dawley rats of both sexes.

### DRUGS AND CHEMICALS

The drugs and chemicals used were: morphine sulfate (Merck Chemical Co.), methadone hydrochloride and 1- $\alpha$ -acetyl-methadol (LAAM) (Eli Lilly Co.), levorphanol hydrochloride and dextrorphan hydrochloride (Hoffman La Roche Co.), naloxone hydrochloride and naltrexone hydrochloride (Endo Laboratories).

### PROCEDURES

#### Effects of Narcotics and Narcotic Antagonists on Volitional Consumption of Ethanol

Animals were housed individually in standard, stainless steel wire mesh cages in a laboratory with an ambient temperature of  $21.0 \pm 0.5$  degrees C and a four hour light and ten hour dark cycle. After at least four days of acclimatization to these conditions, the animals were used for experimentation. Except when otherwise specified, animals had free access to food (Purina Lab Chow), water and/or ethanol (prepared fresh daily from 95% ethanol in distilled water). All fluids were placed in graduated Richter type drinking tubes (100 ml, in the case of rats, or 25 ml, in the case of mice) fitted on the outside of each cage. Daily consumption of each of these fluids was recorded between 11 a.m. and 2 p.m. The tubes were randomly rotated each day to prevent the development of position habit. In most experiments, food consumption and body weight were also monitored daily. Under a free-choice situation, stable baseline consumption of water, ethanol (5% in rats and 10% in C57BL mice) and morphine solution was established for at least four days (usually the last four of seven days) prior to drug treatment. In some experiments, the preference-aversion cut-off concentration of ethanol was determined in an individual rat. The concentration of ethanol was increased by 1% daily until the animal consumed no measurable amount of ethanol on two consecutive days.

### Acute Experiments

The effects of a single injection of various narcotics and narcotic antagonists in doses ranging from 2 to 60 mg/kg on subsequent voluntary consumption of ethanol were studied in both rats and mice.

### Chronic Experiments

Chronic administration of morphine or methadone by injections, or by pellet implantation in the case of morphine, were performed as described previously (11). In one experiment, morphine was dissolved in drinking water at concentrations of 150 mg/l for thirty-one days, followed by 2 g/l for forty days. The differential selection of water, alcohol or morphine solutions was measured at various periods.

Abstinence signs in morphine dependent animals were observed by injecting the narcotic antagonists, naloxone or naltrexone (0.1 or 0.4 mg/kg s.c.), to precipitate narcotic withdrawal symptoms. The abstinence syndrome included body weight loss, spontaneous withdrawal jumping, diarrhea, teeth chattering, ptosis, wet dog shakes, and irritability to handling and touch.

Physical dependence on alcohol was produced by a chronic forced-drinking schedule described previously (11). The abstinence signs due to ethanol withdrawal were observed in rats using a scoring system as follows:

	<u>Score</u>
Loss of 20% or more of the control body weight	1
Mild tremor, hyperreflexia, compulsive drinking	2
Continuous tremor, provoked convulsions (by ringing keys around the animals for one minute)	3
Jumping (provoked by ringing of keys), clonic-tonic convulsions (at least two or more/hour)	4
Severe clonic-tonic convulsions (at least four or more/hour)	5
Coma and/or death	6

Rats were chronically exposed to morphine and methadone and after dependence was established by utilization of naloxone to precipitate jumping, the animals were placed on a forced-drinking alcohol regimen. At various times during the forced drinking schedule, these rats were assessed for withdrawal symptomatology according to the scoring system outlined above. In addition, certain rats were removed from the forced drinking paradigm and allowed to select either alcohol or water according to a three-bottle two-choice method (9).



Thus, withdrawal symptomatology and volitional consumption of ethanol was monitored in animals pretreated with morphine and methadone, and others only saline pretreated.

## RESULTS

### Acute Experiments

#### *Narcotics and Narcotic Antagonists*

Results obtained showed that, in both rats and mice, the acute single injection of active opiates significantly suppressed the voluntary consumption of ethanol in a dose-related manner. The duration of the suppression is only transient, however, as complete recovery to the pretreatment level of ethanol consumption is seen by the next day.

Figure 1 shows the results obtained with various doses of morphine in the C57BL/J6 mice. Water consumption in these mice showed large individual variations. Furthermore, there was no significant alteration in food and water intake or in body weight.

Figure 2 shows the results obtained with methadone in C57BL/J6 mice. Significant depression of ethanol intake was observed with a 30 mg/kg s.c. dose of methadone in the mice. In additional experiments, mice treated with LAAM (2 mg/kg and higher) also experienced a reduction in their alcohol intake. There was significant differences between the acute effects of levorphanol and its inactive stereoisomer, dextrorphan. Significant reduction in ethanol and food intake were observed with 30 mg/kg and 60 mg/kg doses of levorphanol, whereas treatment with dextrorphan at similar doses failed to cause significant change in ethanol, food or water intake, or in body weight (Figure 3).

In Long-Evans rats, similar results were obtained illustrating that alcohol consumption was significantly reduced by subcutaneous doses of 10 mg/kg and 30 mg/kg morphine ( $p < 0.01$ ). With methadone (30 mg/kg s.c.) and LAAM (2 mg/kg and higher) significant depression of ethanol intake was observed. However, rats treated with either naloxone or naltrexone showed a small but not significant increase in ethanol intake (Figure 4). No reduction in food and water intake was demonstrated. To test the selectivity of ethanol, the rats were treated with morphine at various doses and allowed to select between water and sucrose (3%). No significant reduction in the intake of sucrose solution was observed.

Neonatal rats were administered 5 mg/kg morphine starting at day three post-partum and increased by 2 mg/kg/day until a dose of 40 mg/kg/day was attained. This plateau was maintained until morphine administration was terminated on

### Acute Effects of Varying Doses of Morphine on Ethanol Selections in C57Bl/J6 Mice.

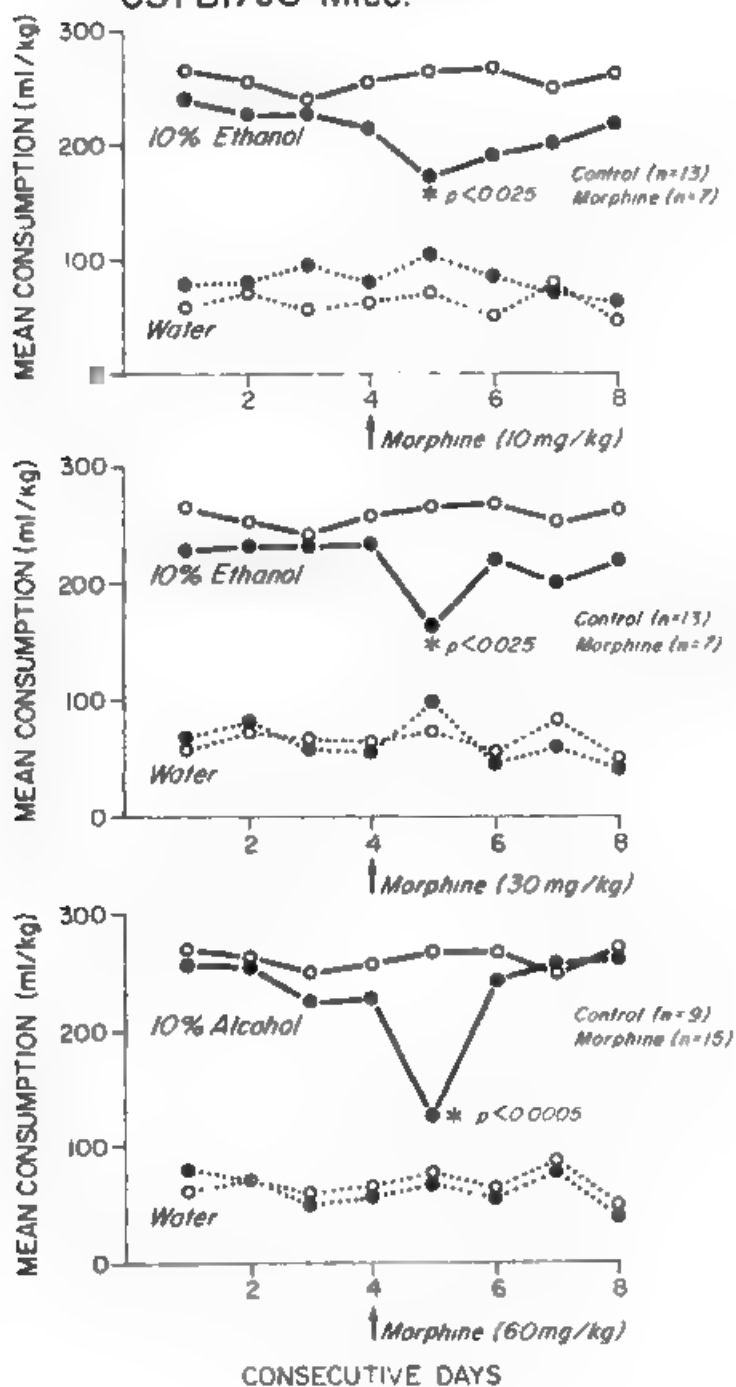


Figure 1

EFFECTS OF METHADONE ON FOOD INTAKE, BODY WT., WATER AND ETHANOL SELECTION IN C57Bl/6J MICE

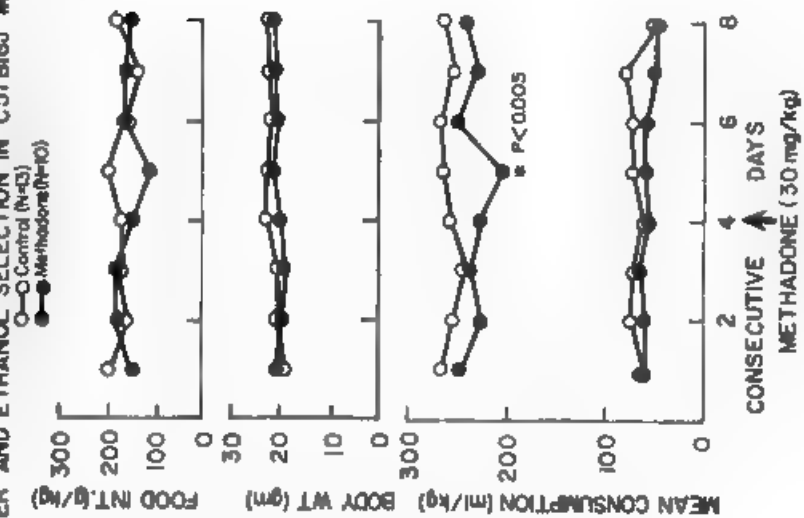


Figure 2

COMPARISON BETWEEN THE ACUTE EFFECTS OF LEVORPHANOL AND DEXTROPHANOL ON ETHANOL SELECTIONS IN C57Bl/6J MICE

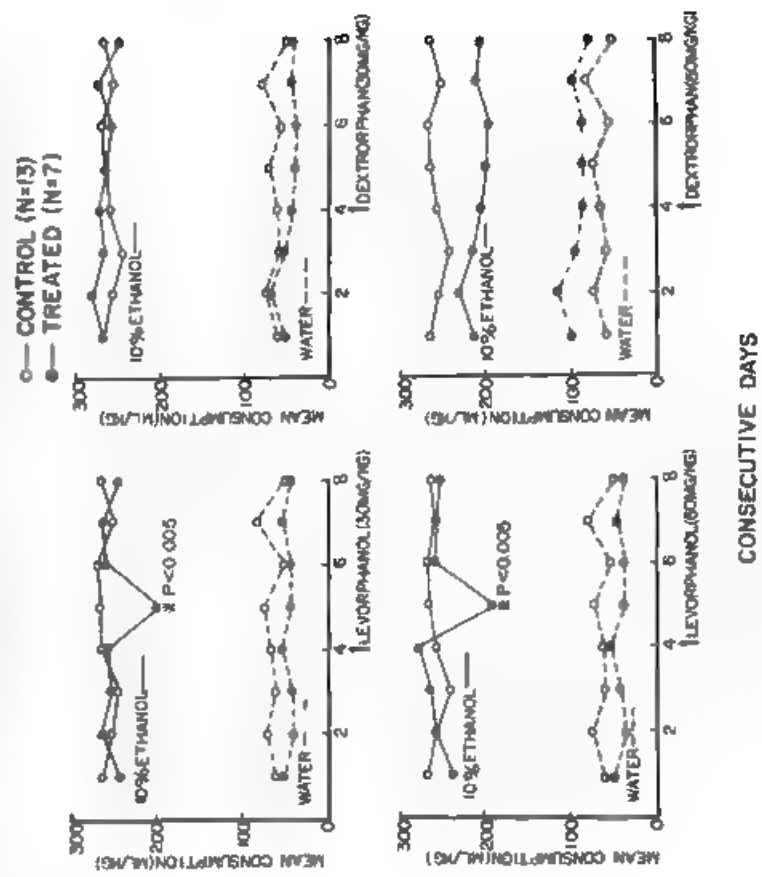


Figure 3

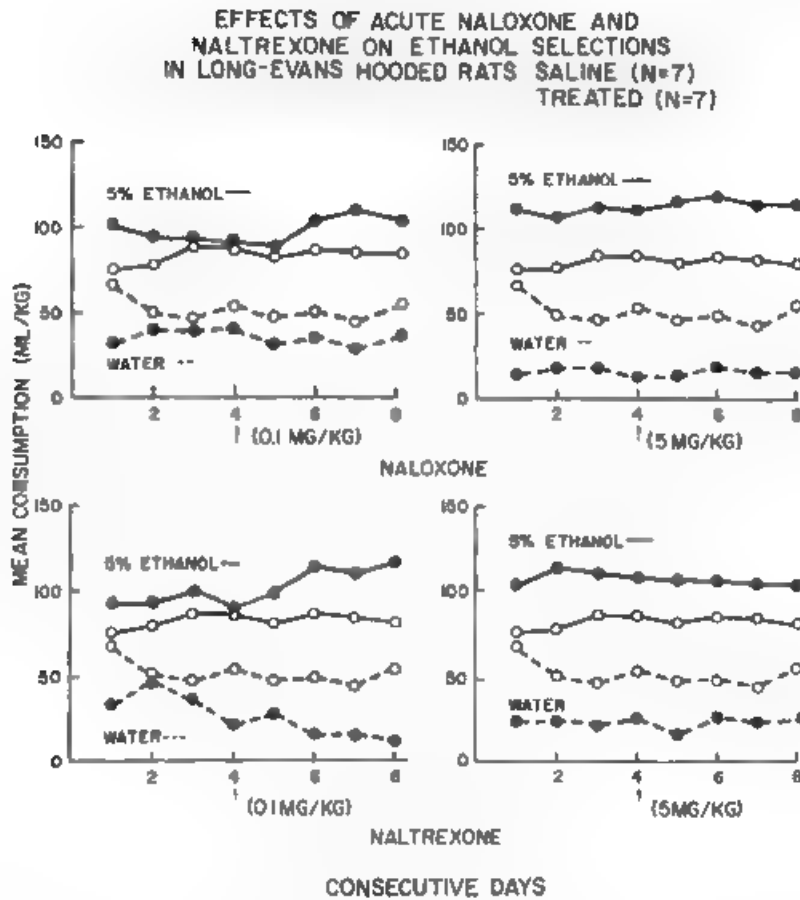


Figure 4

day forty-seven post-partum. After morphine was terminated the rats showed a higher ethanol intake compared to saline controls. Food and water intake were both suppressed in the treated group (Figure 5).

Figure 6 illustrates the effects of morphine withdrawal on the selection of ethanol at different concentrations (5 - 21%). Our results indicate that morphine pretreated animals, as a group, showed a significantly higher preference-aversion cut-off concentration compared to non-treated controls. Between 5 and 7% of morphine pretreated rats selected ethanol compared to less than 75% of the non-treated controls. When the concentration of ethanol was increased to 13% approximately 25% of the morphine pretreated animals continued to select ethanol whereas none of the animals in the control group selected ethanol. Thus, the maximum preference-aversion cut-off for the morphine pretreated group was at 21% compared to 13% for the non-treated controls.

As illustrated in Figure 7, after fifty-one days, both the morphine pretreated and non-treated rats (as previously described in figure 6) were then exposed to ethanol on a

EFFECTS OF MORPHINE WITHDRAWAL ON FOOD  
INTAKE, WATER AND ETHANOL SELECTIONS IN NEONATAL RATS

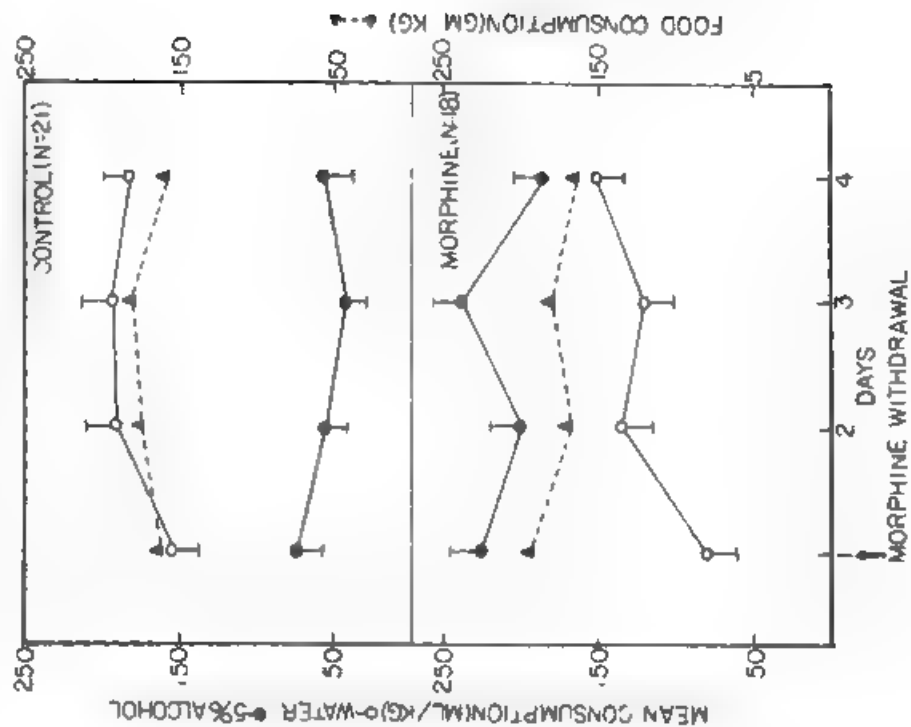


Figure 6

EFFECTS OF MORPHINE WITHDRAWAL ON  
THE SELECTION OF ETHANOL AT DIFFERENT CONCENTRATIONS

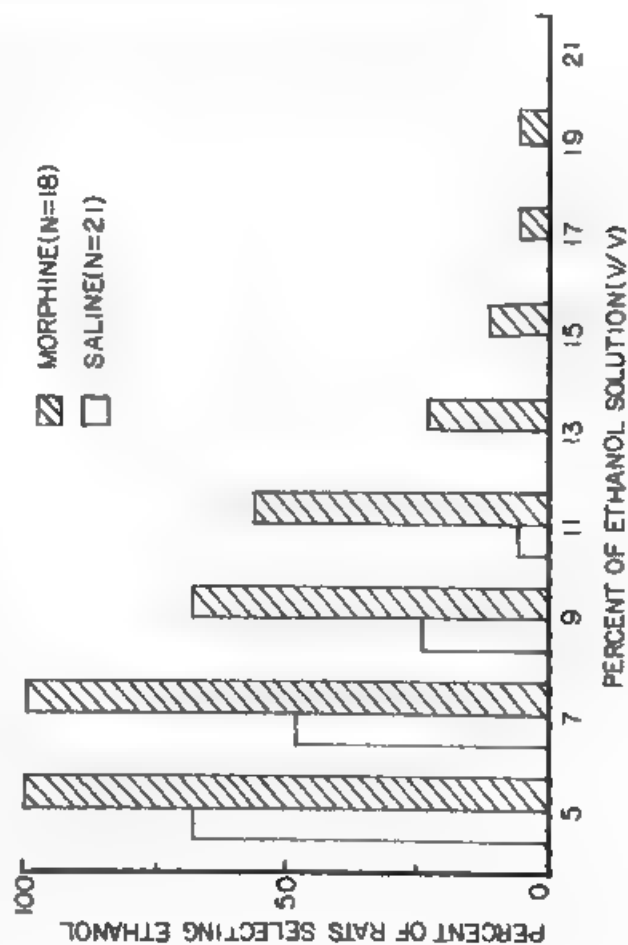


Figure 5

forced-drinking schedule for thirty-two days with a graded increment in ethanol concentrations from 10 to 40%. In comparison with the controls, the morphine pretreated rats consumed significantly less ethanol during this period, however, both groups of rats showed similar food intake. Although we have no explanation for these findings, nevertheless, upon withdrawal from ethanol, the morphine pretreated rats showed a greater withdrawal severity, assessed by provoked withdrawal jumping, hyperreflexia, tremor and convulsions, compared to controls. In this regard, after the thirty-two day forced drinking of alcohol, over 50% of the morphine pretreated rats showed jumping, whereas none of the controls showed provoked jumping.

Figure 8 illustrates that after two months of forced ethanol consumption morphine pre-treatment still exacerbated withdrawal scores. Note, however, that some 10% of the control rats showed provoked jumping, a withdrawal symptom that was not evident in the one-month control animals.

In another experiment, two groups of adult rats were similarly treated with morphine (as previously described for the neonatal rats) for six weeks. One group of the animals were allowed free choice between water and quinine solution ( $4 \times 10^{-4}$  %) and the other group was given free choice between sucrose (3%) and a mixture of sucrose (3%) and ethanol. No significant difference was observed between the morphine treated and the controls in the selection between water and

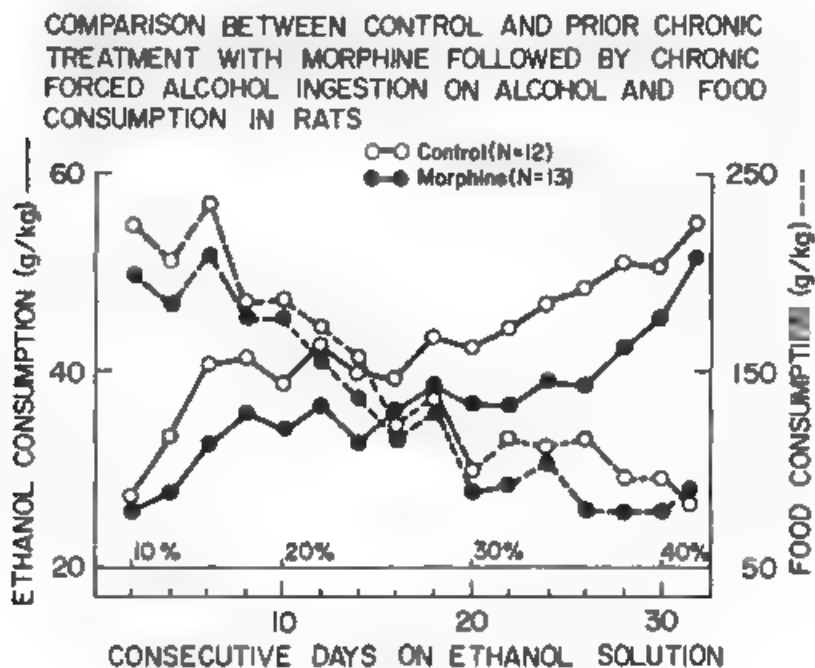


Figure 7

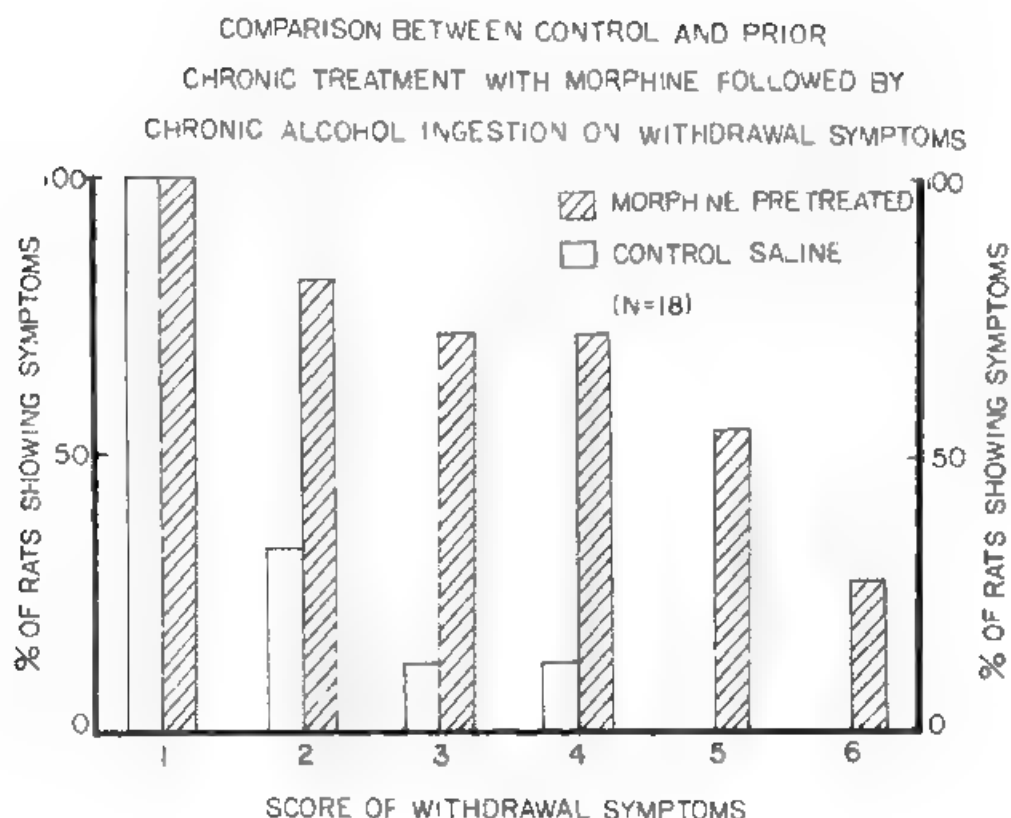


Figure 8

quinine solution. Both groups of rats showed preference for water. On the other hand, the morphine treated rats showed a marked preference for alcohol-sucrose mixture whereas the controls showed a marked preference for sucrose solution (Figure 9).

### Chronic Experiments

#### *Effect of Morphine Withdrawal on Ethanol Selection*

In this study, adult Sprague-Dawley rats were made dependent by chronic injection of morphine according to the schedule outlined previously for the neonatal rats. To test dependence, some of the rats were injected with naloxone to precipitate characteristic withdrawal symptoms. Rats treated in the above manner were shown to be dependent by day fourteen as evidenced by naloxone-induced jumping, weight loss, diarrhea and wet dog shakes. Our results indicate that there was a decrease in the daily intake of food and water during withdrawal. Ethanol consumption was not depressed, but showed an increase during the four days after morphine withdrawal.

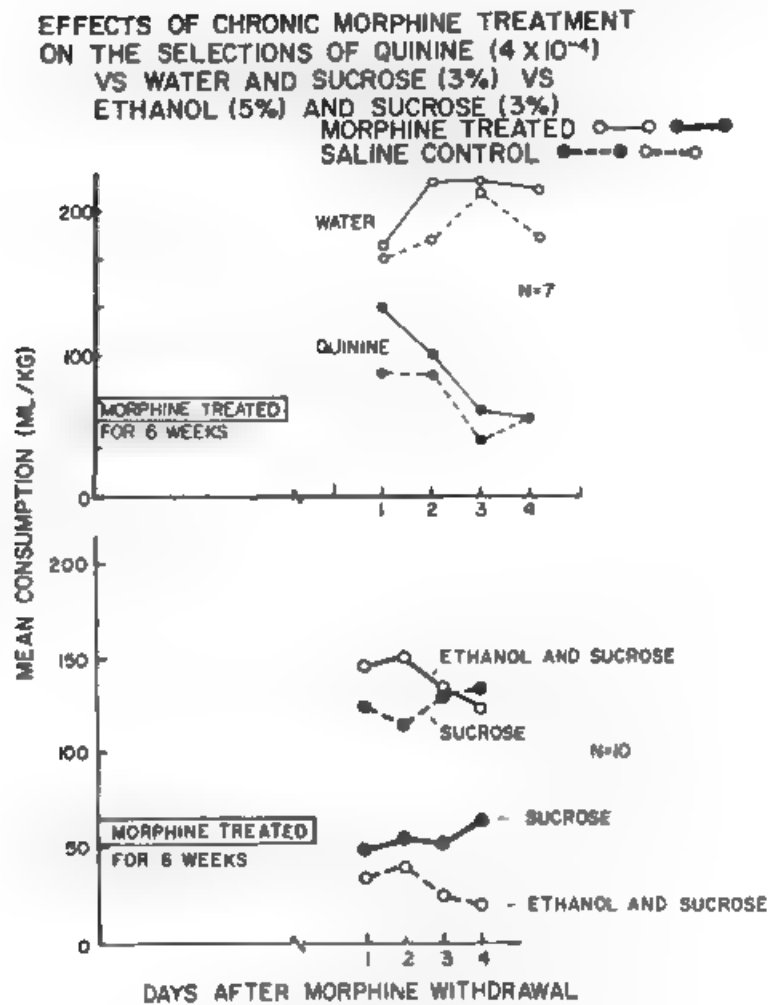


Figure 9

#### *Effect of Ethanol Forced Drinking on Ethanol and Morphine Selection*

Rats were exposed to a forced-ethanol drinking paradigm over an eighty-six day period. During this exposure, at certain specified intervals, the animals were removed from the forced drinking condition and allowed to select either water, morphine and ethanol freely. Our results demonstrate that the animals showed a significant increase in the selection of both morphine solution and alcohol (5%) at the 65th and 82nd days.

#### DISCUSSION

The results presented in this study tend to confirm our earlier reports that narcotics interact with ethanol after the acute and chronic administration and during the withdrawal



state. The fact that narcotics suppressed the voluntary consumption of ethanol after the acute administration and not after chronic treatment, suggests the development of tolerance in the rats to this effect. Thus, a single injection of morphine, methadone, LAAM, or levorphanol, but not dextrophan or naloxone, significantly suppressed the voluntary consumption of ethanol in rats, mice or hamsters (9-11). There appears to be some specificity in the opiate-treated animals for ethanol. Dextrophan has no effect in the volitional consumption of ethanol or blood ethanol level.

It is of interest to note, that rats were able to discriminate ethanol from ethanol-sucrose mixture, and a quinine or sucrose solution from water. Animals furthermore were given free access to food, water and ethanol and there was no schedule-induced influence on the daily consumption of ethanol. The interactions seen after chronic opiates and chronic ethanol are rather striking. The rats treated chronically, as neonates, with morphine or methadone, followed by forced drinking of ethanol, showed lower ethanol intake than untreated controls. These same animals, however, showed more severe and more rapidly developing withdrawal signs after ethanol withdrawal. This may be due to an additive effect on dependence liability, in that the rats previously treated with opiates subsequently require less ethanol to reach a state of ethanol dependence. There appeared in the literature several studies in support of opiate-ethanol interaction(s). Blum et al. (12) reported that naloxone significantly inhibited ethanol induced dependence in mice. This same group in another study (13) also found that morphine administration, significantly suppressed ethanol induced withdrawal symptoms in mice utilizing the Goldstein-Pal (14) inhalation technique. Unlike morphine, dextrophan was not active in ameliorating these withdrawal reactions in mice (15). Ross et al. (16) showed that in hamsters, both morphine and levorphanol, but not dextrophan, suppressed alcohol consumption, and this is in agreement with our findings in mice and rats. Along similar lines Blum and co-workers have further advanced the possibility of a common mechanism between ethanol and opiate dependence (17) and suggested that the common link between these two addictive substances may be the dopamine-derived isoquinoline, salsolinol. Furthermore, the thesis proposed by Cohen and Collins (18) concerning the possible involvement of salsolinol, in both ethanol effects and post-ethanol intoxication states has received support from other investigators (19,20).

The lack of tolerance to morphine in mice and humans previously made dependent on ethanol, as evidenced by the increase in lethality under the influence of both opiates and ethanol (3,7) may be partly explained by neurochemical alterations induced by these two commonly abused psychoactive

chemicals. Our findings concerning the enhanced severity of withdrawal in animals after prolonged exposure to both morphine and ethanol may be either due to common neurochemical and behavioral mechanisms as suggested in the review by Blum et al. in this volume or to effects on metabolism. With regard to the latter, we are currently looking at penetration of the opiates into the CNS. The fact that, in the rats treated with higher doses of morphine or methadone, the retention of alcohol in the blood is markedly prolonged (11), may be related to an overall increase in the depression of both the circulatory and respiratory systems. There appears to be some circumstantial evidence, from autopsy findings, indicating the presence of high levels of either alcohol or the opiates, or both, in drug related deaths (3). This warrants a more systematic investigation in animals in order to gain further insight into the potential hazards of interactions between opiates and alcohol.

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## 12. ALCOHOL AND OPIATES: A REVIEW OF COMMON NEUROCHEMICAL AND BEHAVIORAL MECHANISMS

Kenneth Blum, Murray G. Hamilton and J.E. Wallace

The University of Texas Health Science Center at San Antonio,  
Departments of Pharmacology and Pathology, San Antonio, Texas,  
78284, and The University of Western Ontario, Department of  
Pharmacology, London, Ontario, Canada.

### INTRODUCTION

Among drugs of abuse, none have achieved such wide popularity as ethanol and opiate derivatives. Although these drugs are consumed by a large segment of our population, many consequences of acute and chronic intoxication with alcohol and opiates are not readily understood.

Alcohol, on one hand, is a central nervous system depressant, as are the opiates, but differs from them in that it is a nutrient whose rapid oxidation in the liver causes marked disruption of normal metabolic processes. There are differences between these two commonly abused drugs when chronically consumed: alcohol is more cytotoxic than opiates and potentially more dangerous when abruptly removed. Other differences include effects on the autonomic nervous system, electrical activity of certain pathways in the brain, and antinociceptive response (1,2).

The widespread abuse of opiates and ethanol in modern society has resulted in tremendous scientific investigation into the mechanisms of action of these two diverse classes of drugs. Although it is well known that one drug is often associated with concurrent abuse of the other (3-7), there has been little effort devoted to establishing a common underlying biochemical mechanism between the two. This is not surprising, as it is difficult to envisage the biochemical, physiological or metabolic pathways that complex phenanthrene-type alkaloids would have in common with a simple 2-carbon molecule.

In this chapter we shall attempt to evaluate the "state of the art" concerning a biochemical link between opiates and ethanol. Thus, we will deal with behavioral and neurochemical similarities and finally an evaluation of the possible role of tetrahydroisoquinoline (TIQ) alkaloids in modulating these effects.

The results that follow demonstrate the numerous attempts have been made to define the locus of the behavioral and the tolerance- and dependence-producing properties of opiates and ethanol. However, caution must be used in interpreting these results because of the complex interrelationships among various neural elements and their respective neurotransmitters. Thus, while a particular agent is considered to affect only one system, and while biochemically that may indeed be true, the loss (or increase) of that specific system may inhibit or excite several others. Nevertheless, studies of this nature are extremely important in elucidating not only the neurochemical and behavioral mechanisms of opiates and ethanol, but also in unravelling the complex interrelationships that exist in the nervous system.

#### BEHAVIORAL SIMILARITIES

##### Opiate-Ethanol Interaction Studies

There is considerable evidence in the literature indicating a relationship between opiates and ethanol. There are reports of acute interactions between these substances in both humans (8) and experimental animals (9,10). Sinclair (11) has shown that morphine suppresses voluntary alcohol consumption (VAC) in hamsters and Ho *et al.* (12) have demonstrated that a single injection of an opiate agonist significantly suppresses VAC in both mice and rats. Ross *et al.* (13) have reported the same effects in hamsters and have also shown that dextrorphan had no significant effect on alcohol consumption indicating the necessity for an active opiate enantiomer.

In another study (14), single injections of naloxone or naltrexone produced a slight but non-significant increase in VAC in hamsters six to eight days following a single injection of 2.5 or 5 mg/kg naltrexone. It is difficult to understand the delay in the response after the injection of opiate antagonist, as the half-life of the drug is considerably shorter than six to eight days. Nonetheless, the effect is reproducible and may relate to heretofore unappreciated pharmacological aspects of the drug.

Chronic administration of morphine to adult rats for fourteen days produces an increase in VAC during morphine withdrawal (11). Similar results were obtained during withdrawal with neonatal rats treated with morphine for forty-four days. Further evidence for a relationship between ethanol and the opiates has been obtained through the use of the narcotic antagonists naltrexone and naloxone to ethanol-dependent animals. It is well recognized that both of these drugs are capable of blocking the acute effects of opiate agonist and the development of tolerance and dependence to

opiates (15). The first experiments with narcotic antagonists in ethanol-dependent animals showed that administration of naloxone to ethanol-dependent mice did not precipitate jumping behavior, as it does in opiate dependent mice (16). However, recent experiments (17) show that concurrent administration of naloxone during ethanol-vapor exposure significantly attenuates the resultant withdrawal convulsions. As well, naloxone or naltrexone at 5 mg/kg can inhibit ethanol narcosis in mice, while higher doses of the narcotic antagonists (10 mg/kg) potentiate the narcosis (18).

Additionally, Blum et al. (19) have shown that the ethanol-withdrawal syndrome in mice is significantly suppressed by a single injection of 10 mg/kg morphine at the fifth hour post-ethanol exposure. Jones and Spratto (20) have recently demonstrated that concurrent administration of ethanol can affect morphine withdrawal in rats, and Uyeno (21) has observed that withdrawal of ethanol after chronic administration increases morphine self-administration in monkeys.

## CONCLUSION

While these studies indicate that there may be similarities between opiates and ethanol as far as behavioral parameters are concerned, there is no evidence that the same, or even similar, mechanisms are involved. The following sections will discuss the effects of intoxication caused by alcohol and opiates on basic neurochemical organization in the CNS and, also, the effect of prior manipulation of endogenous neurohumoral amines on the behavioral consequences of administration of these agents.

## NEUROCHEMICAL SIMILARITIES

Both opiates and ethanol produce alterations in neuroamine metabolism and concentrations. These effects occur after acute administration, during chronic administration and also during the withdrawal phase.

### Norepinephrine (NE)

Morphine has been shown to decrease (22,23), increase (24), or have no effect (25) on NE concentrations. Similar results have been reported for ethanol, that is, decreases (26), increases (27), or no effect (28). The conflicting results obtained concerning NE concentrations may reflect differences in the sensitivity of the techniques employed, the timing of NE measurement, the doses or the species and thus prevent any definite conclusions regarding similarities between opiates and ethanol after acute administration.

Although the effect of an acute dose of ethanol on NE is uncertain, there is general agreement that during chronic administration ethanol induces an increase in NE turnover (29-32) and concentration (32). This same effect is also evident in chronically morphinized rats (24,33,34), although no effect on NE was observed in morphinized dogs (35).

During withdrawal from chronically administered opiates, NE levels have been reported to decrease in dogs and rabbits (23,33,35), while there is no apparent change in the NE levels in rats subjected to either abrupt or precipitated (nalorphine) withdrawal (33,36). Withdrawal from ethanol generally produces an increase in NE turnover in rats (32) and mice (37). In rats, the increase in NE turnover is maintained even when withdrawal signs are no longer evident (32).

#### Dopamine (DPA)

There is considerably less information concerning the effects of opiates and ethanol on dopamine (DPA) concentration and metabolism than that pertaining to NE.

Nonetheless, acute injections of 20 mg/kg morphine to mice has been shown to decrease DPA levels (22) and this depletion is blocked by naloxone. However, another report (38) indicates that 40 mg/kg morphine had no effect on DPA levels in mice. Similar results, that is decreases (39) or no change (30) in DPA levels have been reported following acute ethanol administration.

Chronic administration of morphine increases DPA turnover in rats (25) and chronic ethanol produces increases (30), decreases (33,35), or no effect (40) on DPA turnover, whereas central DPA concentrations after chronic administration of ethanol are reported to be unaffected (30,40). While withdrawal of opiates from dependent rats (33,41) and dogs (35) produces decreases in DPA concentrations little information on the effect of ethanol withdrawal on DPA concentration is presently available from animal studies. However, Gitlow et al. (42) reported elevated excretions of the DPA catabolites homovanillic acid (HVA) and 3-methoxy-tyramine (3-MT) in human subjects withdrawing from ethanol. These results suggest that ethanol modifies the metabolism of DPA under these experimental conditions.

#### 5-hydroxytryptamine (5-HT)

Acute administration of opiates increases (43), decreases (44) or have no effect (45) on 5-HT concentrations. Similar results have been reported after acute ethanol injections (26,44,46). Chronic morphinization has no effect (47) on 5-HT concentrations and chronic ethanol administration has

not been found to affect 5-HT levels (48). The results obtained during withdrawal from opiates or ethanol are similarly contradictory with increases (37), decreases (37,49), and no effect on 5-HT concentrations (23,44,49,50).

#### Gamma-Aminobutyric Acid (GABA)

Attempts have been made to correlate the actions of morphine with alterations of content and/or metabolism on  $\gamma$ -aminobutyric acid (GABA), a putative inhibitory neurotransmitter in the central nervous system. Ho et al. (51) demonstrated that administration of GABA enhances the development of morphine tolerance and dependence in mice and Yoneda et al. (52) found that GABA may be involved in morphine analgesia, although single injections of morphine do not affect brain GABA content in rodents (52,53). However, based on the work of Yoneda et al. (52), functional alterations of the GABA system in the CNS may also be an important factor for the occurrence of both acute and chronic actions of morphine.

GABA and ethanol possess anticonvulsant properties (54) and thus a number of investigators have attempted to establish a relationship between these two compounds (55).

Ethanol, unlike morphine, was reported to increase GABA levels in rat brain *in vivo* (56). Ethanol-induced elevation of brain GABA, was also found by other investigators in rats (57) and cats (58); but some investigators found no change or a decrease (59,60). The apparently conflicting results were not reconciled on the basis of strain differences (61), inadequate nutrition (62), subcellular distribution, (63) or mode of administration.

It is possible that GABA may play a role in both acute and chronic effects of ethanol, as postulated for morphine, but any definitive conclusions on common mechanisms with regard to this is premature.

#### Acetylcholine (ACH)

Administration of morphine causes an increase in ACH concentrations in rodents (64,65,66). It has also been shown to increase the amount of "bound" ACH while decreasing the amount of "free" ACH (67). Similar increases in ACH levels of rat brain have been reported with acute injections of both ethanol and acetaldehyde (66,69) and acetaldehyde (70) in frog brain.

Chronic administration of ethanol for twenty-two weeks decreased rat brain concentrations of ACH (71) and Ho (72) found a reduced amount of brain ACH in rats made dependent following a two month forced feeding regiment. However, a five week course of ethanol to guinea-pigs did not affect



ACH levels (73).

Adenosine 3'5'-Monophosphate (cyclic AMP)

Contradictory findings concerning the effects of acute administration of both opiates and ethanol on brain cyclic AMP levels have been reported. Acute injections of opiates and ethanol have been found to increase (74,75,76), decrease (75, 76) or produce no change (77,78,79). However, chronic intoxication with ethanol has been found to affect the cyclic AMP system in the brain. Israel *et al.* (80) reported that administration of ethanol in a liquid diet for two weeks increased adenylate cyclase activity in mouse brain cerebral cortex. Similarly, increases in basal cerebral adenylate cyclase activity was obtained with chronic administration of morphine (81).

During ethanol-induced withdrawal French and Palmer (82) reported that the cyclic AMP system is affected by ethanol withdrawal. These authors incubated brain slices with NE and found that formation of labelled cAMP from prelabelled ATP was higher in cortical slices of rats withdrawn from an ethanol-containing diet than in slices from control animals. Similarly, Mehita and Johnson (83) reported an elevation of brain cAMP during naloxone precipitated withdrawal in morphine dependent rats.

These results suggest that the sensitivity of adenylate cyclase, and thus formation of cAMP, may play an important role in dependence on, and withdrawal from ethanol and opiates.

CONCLUSION

It is apparent from the results reported in the preceding sections that there is little agreement concerning the effect that ethanol or opiate intoxication and withdrawal can produce on neurochemical mechanisms.

The conflicting results obtained are most likely related to a combination of factors, in the experimental protocols, including different routes of administration, different monoamine measuring techniques and/or the timing of the measurements, or the difference in the utilized doses to produce the desired effect (acute intoxication or dependence). While this list is by no means exclusive, it does point out several reasons why there is no general agreement concerning the neurochemical alterations elicited by either ethanol or opiates.

The lack of agreement in this area with either of these substances above, obviously precludes a definite conclusion concerning similarities, or differences, in the neurochemical alterations induced by acute or chronic treatment.

## **EFFECT OF MONOAMINE ALTERATION**

Further evidence for a common mechanism of action has been obtained through pharmacological manipulation of central monoaminergic function. Considerable research has implicated changes in brain biogenic amines, induced by opiates and ethanol administration, as mediating at least some of the effects of acute or chronic drug intoxication. Although the exact mechanism and direction of changes in biogenic amines induced by ethanol and opiates remains controversial, that changes occur is generally accepted. This has prompted several investigators to examine the effects of biogenic amines on behavioral changes induced by these two drugs. Here we review the results of investigations of neurochemical alterations on opiate and ethanol-induced tolerance, dependence and withdrawal and attempt to point out possible common mechanisms.

### **Dependence and Tolerance**

#### ***Effect of Protein Synthesis Inhibitors***

The possibility that either opiates or ethanol induced tolerance, or physical dependence, or both, may result from modification of a macromolecule in the brain has been considered by several laboratories. Cochin (84) reported that inhibitors of protein synthesis blocked the development of tolerance to morphine. Loh and associates (85) found that the development of physical dependence on and tolerance to morphine can be inhibited in the mouse by the concomitant administration of cycloheximide. These investigators also found that cycloheximide, while inhibiting development of tolerance did not alter analgesic response to morphine (85).

Similar findings concerning the effects of protein synthesis inhibition on tolerance and physical dependence induced by administration of ethanol have been reported. LeBlanc and associates (86) found that cycloheximide inhibited behavioral adaptation (tolerance) to ethanol and the development of physical dependence to ethanol in mice can be markedly reduced by the concomitant administration of large doses of cycloheximide (87).

These results suggest that *de novo* protein synthesis is involved in the development of tolerance and physical dependence to both opiates and ethanol. It does not mean, however, that the same macromolecule is involved in both cases, and it is also possible that the effects observed with cycloheximide are not due to inhibition of protein synthesis at all, but rather to some other "side-effect" of the drug.

#### ***Effect of Neurotransmitter Alterations***

Various pharmacologic agents have been utilized to affect, as selectively as possible, the synthesis, storage, release or degradation of NE, DPA, 5-HT, GABA, ACH and cAMP during administration of opiates and ethanol. The consequences of such modification of amine activity on the tolerant-dependent and withdrawal states will be reported in the following sections.

*Catecholamines (CA's).* Based on numerous investigations, it appears that catecholamines participate in the acute pharmacologic effects of morphine (88) and in signs and symptoms observed during withdrawal (89). Martin and Eades (90), Way et al. (91) and Blasig et al. (92) have suggested that CA's are not primarily involved with either tolerance or physical dependence development, but that NE may be related to morphine antinociception. Experiments with mice rendered tolerant-dependent by morphine pellet implantation showed that 6-OHDA-induced decreases in brain NE and DPA concentrations does not effect the development of either tolerance or dependence, but the 6-OHDA pretreatment was accompanied by a decrease in analgesic response to morphine (i.e. an increase in the morphine  $AD_{50}$ ). This was apparent to the same degree in both the non-tolerant animal and the tolerant animal as well. Blasig et al. (92) found that when CA's were kept low throughout the morphine exposure and also at the time of withdrawal, the intensity of withdrawal, as measured by jumping and wet dog shakes, was not significantly affected. At the same time though, there was a great increase in the frequency of another withdrawal sign, writhing.

Other experiments suggest that the non-involvement of catecholaminergic systems in morphine tolerance and dependence is not so clear cut. Administration of the  $\beta$ -adrenergic receptor blocking drug, dichloroisoproterenol, significantly attenuated the development of tolerance and physical dependence in mice (88). Furthermore, Huidobro et al. (93) found that concurrent administration of Dopa to morphine treated rodents attenuated the intensity of the resultant abstinence syndrome precipitated by injection of naloxone.

Experiments dealing with the role of CA's on ethanol induced tolerance and dependence reveal that  $\alpha$ -adrenergic function may play an important role in the development of the tolerant-dependent state. It has been reported that agents that reduce (functionally or through depletion) catecholamine activity throughout ethanol exposure produce an exacerbation of withdrawal (94,95). Agents studied included  $\alpha$ -methyl-p-tyrosine (AMPT), phenoxybenzamine (POB), and 6-OHDA and the findings suggest that dependence is antagonized by central catechoaminergic mechanisms, and that interference with these mechanisms, through reduction of receptor-mediated antagonism produces a greater degree of dependence and thus higher withdrawal scores. Ritzmann and Tabakoff (96) obtained

evidence which indicates that an intimate relationship exists between the noradrenergic system and the development of tolerance to ethanol after chronic treatment. These authors found that control animals consuming an ethanol-containing diet became quite tolerant to the effects of ethanol, while the animals treated with 6-OHDA prior to chronic ethanol administration did not develop any tolerance to the temperature lowering or hypnotic effects of ethanol. In these experiments, no difference in withdrawal symptomatology was evident between the 6-OHDA-treated animals and the control physically dependent animals indicating that 6-OHDA did not affect the development of physical dependence.

Similar to morphine, results from our laboratory (95) indicate inhibition of physical dependence development to ethanol with (d,l)-propranolol, a  $\beta$ -adrenergic blocker. There are at least two possible mechanisms to consider: i) a chronic blockade of  $\beta$ -receptors which reduces the development of dependence to ethanol, or ii) the membrane stabilizing effects of (d)-propranolol (97) which protects central neurons from the disruptive membrane effects of ethanol.

The demonstration that propranolol prevents the cerebral cortical cAMP response to NE *in vitro* (82) suggests that hyperactivity induced by ethanol withdrawal may be mediated through  $\beta$ -adrenergic pathways (82). If the supersensitivity to NE observed in the studies by French and co-workers (82,98) reflect an increase in the  $\beta$ -receptors located on the post-synaptic membrane, this may provide an explanation for the beneficial effects observed with propranolol in our studies and during the abstinence phase of chronic alcoholics (99, 100). However, these findings are not in agreement with the observation by Goldstein (116) that propranolol administered to mice undergoing abstinence from ethanol, produced a slight exacerbation of withdrawal convulsion scores. Nevertheless, which of these mechanisms is operant remains speculative, but we feel the second alternative represents a distinct possibility and warrants further investigation.

In addition, similar to the findings of Huidobro et al. (93), daily administration of large doses of L-dopa (620 mg/kg) during ethanol vapor exposure to mice significantly attenuated ethanol induced dependence suggesting possible involvement of DPA (101). Thus, it is of interest that Iwamoto et al. (102) reported that the stereotyped jumping which occurs in morphine-dependent mice or rats after abrupt or naloxone-precipitated withdrawal may depend upon a sudden elevation of brain DPA levels. When naloxone was given to mice and rats rendered dependent on morphine by pellet implantation, brain levels of DPA, but not those of NE or 5-HT, increased 20% to 40% above control levels within five minutes, a time that corresponds to the peak in

precipitated stereotyped jumping. Catecholamine synthesis inhibition with AMPT partially blocked the increase in DPA after naloxone and increased the amount of naloxone required to induce jumping. Furthermore, elevation of ACh by cholinesterase inhibition with physostigmine blocked the sudden rise of DPA levels as well as the jumping response. This suggests that cholinergic-dopaminergic pathways may mediate the jumping response of naloxone-precipitated withdrawal.

*Serotonin (5-HT).* The exact role of 5-HT in the morphine tolerant-dependent state is still controversial despite a large amount of interest and work in this area. Juidobro et al. (93) have found that administration of 5-HT during the development of morphine dependence had no significant effect on subsequent naloxone-precipitated withdrawal. However, in the same study, administration of the 5-HT precursor tryptophan during dependence development reduced the severity of the resultant abstinence syndrome. In contrast, to these studies, Ho et al. (103) have reported that morphine pellet implanted mice show a higher degree of tolerance and physical dependence with concomitant tryptophan administration.

Pretreatment with para-chlorophenylalanine, a relatively specific and long-lasting inhibitor of 5-HT biosynthesis, demonstrated both reduced tolerance and physical dependence to morphine in mice and rats. Similar results (104) have been reported using intracerebrally administered 5,6-dihydroxytryptamine, a substance reported to selectively destroy tryptaminergic nerve endings (105).

In mice rendered dependent on ethanol by inhalation of ethanol vapor, concomitant treatment with pCPA had no significant effect. As well, there are no consistent and reproducible effects of administration of 5-HT (intracerebral), 5-hydroxytryptophan (i.p.) (99) or methysergide (i.p.) (106) during the induction of ethanol dependence. However, Collier et al. (107), monitoring a different withdrawal sign (head twitches), found that both pCPA and parachloroamphetamine reduced the degree of ethanol dependence.

The conflicting results obtained with 5-HT, its precursors or inhibitors on either the opiate or ethanol induced tolerant-dependent state preclude a definite role for 5-HT as yet, in these two syndromes.

*Acetylcholine (ACh).* Inhibition of cholinesterase (e.g. with physostigmine) was found to decrease the morphine ED<sub>50</sub> for analgesia in mice (108). However, this effect was evident to the same degree in both morphine tolerant and non-tolerant animals. Additionally, cholinesterase inhibition did not significantly modify either the development of tolerance or physical dependence to morphine (108). These results suggest that ACh does not play a primary role in the tolerance or dependence to opiates. It is possible, though, that ACh may

modulate morphine antinociception, perhaps through an Ach-NE-linked pathway.

A review of the literature reveals little or no available data concerning the effect of modification of central Ach levels on ethanol-induced tolerance or dependence.

*γ-Aminobutyric Acid (GABA)*. There is increasing evidence that GABA acts as an inhibitory neurotransmitter in the CNS (109). This substance was found to antagonize morphine antinociception acutely in the mouse, but with repeated administration it also accelerated the development of both morphine tolerance and physical dependence (88). Amino-oxyacetic acid, which inhibits the transamination of GABA, (i.e. degradation to glutamine) (52) had similar effects. Bicuculline, a GABA-receptor blocker, inhibited both the development of tolerance and dependence to morphine (88,109).

There are few reports of the effects of GABAminergic pathways on the acute neuropharmacological actions of ethanol (110). It is known that a GABA metabolite, *γ*-hydroxybutyric acid, potentiates ethanol induced sleep and produces sleep in its own right (55), but, in contrast to morphine, little or no information exists with regard to the role of GABA in the development of tolerance and dependence to ethanol. Thus, any conclusions with respect to GABA-mediated common mechanisms between ethanol and morphine would be premature.

*Cyclic AMP (cAMP)*. Because of the well-documented (111) relationship between cAMP and virtually all the putative neurotransmitters, the effect of this agent on the development of tolerance and physical dependence to opiates or ethanol presents some difficulties. However, there have been some reports of the effect of functional increases in cAMP on morphine tolerance and dependence.

Intercerebroventricular administration of cAMP antagonized the analgesic effect of morphine (i.e. increased morphine AD<sub>50</sub>) (112); similar results are obtained with dibutyl cAMP and theophylline (113). Chronic treatment of morphine pellet implanted mice with cAMP for three days doubled the morphine AD<sub>50</sub> when compared to placebo implanted mice (114). The suggestion that cAMP increases the degree of tolerance of these animals to morphine is demonstrated by the fact that pretreatment with 10 mg/kg i.v. cAMP before morphine pellet implantation significantly increased morphine AD<sub>50</sub> compared to non-pretreated animals (114).

As well, pretreatment with cAMP two hours before morphine pellet implantation increased the degree of dependence developed in these animals as evidenced by a decrease in the amount of naloxone required to precipitate withdrawal, and a two to three-fold increase in weight loss on withdrawal (114). This same study demonstrated that concomitant administration of cycloheximide antagonized the effect of cAMP (114). However,

in this experiment cycloheximide by itself did not produce any significant effect on the development of dependence, in contrast to earlier reports (85), making these results somewhat difficult to interpret.

There are few similar reports in the literature concerning the effects of cAMP on the development of tolerance and dependence to ethanol. In a preliminary experiment, theophylline administered to mice each day during exposure to ethanol vapor did not have any significant effect on the development of dependence (115).

### Withdrawal

The purpose of this section is to relate the results of experiments which provide insight into the underlying neurochemical mechanisms of the abstinence syndrome following chronic ingestion or administration of ethanol and opiates.

A search of the literature reveals that a greater number of papers deal with the effect of drugs which modify withdrawal states induced by ethanol rather than opiates (88,136). Therefore, withdrawal from ethanol and its modification will constitute the major portion of this section; but drug-induced changes in opiate withdrawal will be considered where data is available.

### *Effect of Neurotransmitters*

Various neuropharmacological tools have been employed to effectively alter the central level and activity of biogenic amines. It is our purpose in this chapter to delineate what effect(s) modifying biogenic amines have on the abstinence syndrome obtained from abrupt removal of ethanol or opiates. The biogenic amines considered will be CA's (NE and DPA), 5-HT, ACH, GABA and cAMP.

CA's (NE and DPA). Goldstein (116) found that several drugs that interfere with CA's facilitated ethanol-induced withdrawal seizures. Blockade of CA receptors at the 5th hour after termination of ethanol exposure, with phentolamine ( $\alpha$ -blocker) or propranolol ( $\beta$ -blocker) produce a transient increase in withdrawal severity (116). Mice treated with AMPT, again at the 5th hour post-ethanol, demonstrated a slight exacerbation of withdrawal convulsions. Reserpine was most dramatic in its ability to increase seizure scores; however, this effect of reserpine is complicated by the finding that normal mice treated with single injections of reserpine showed the same characteristic convulsions on handling as mice undergoing alcohol withdrawal reactions (117). Collier et al. (107) have reported the effects of drugs affecting CA mechanisms on head twitches in mice induced by withdrawal of ethanol.

In these studies, both DPA and NE inhibited ethanol withdrawal head twitching, as did L-dopa. Other drugs that had sympathomimetic activity such as apomorphine (DPA-agonist) and amphetamine (CA releaser) also lessened withdrawal head twitching, whereas AMPT, which inhibits endogenous biosynthesis of DPA and NE (107), increased it.

Other studies (95,120) have extended these findings through the use of various CA agonists and receptor blockers. Although for the most part these studies agree with both Goldstein (117) and Collier et al. (107) there are differences.

Results from our experiments reveal that intracerebral injections of NE, and Clonidine, a central  $\alpha$ -receptor activator (118,119), during ethanol withdrawal exacerbates the ethanol induced withdrawal convulsions (95). In contrast, a similar injection of DPA produced a very marked inhibition (i.e. amelioration) of the withdrawal reaction (120).

Based on these findings, it appears that activation of central  $\alpha$ -receptors (noradrenergic) exacerbates ethanol-induced withdrawal convulsions, while central stimulation of DPA receptors ameliorates withdrawal. The suggestion that DPA ameliorates withdrawal is supported by the demonstration that haloperidol, a central DPA-receptor blocker (121) exacerbates ethanol-induced withdrawal convulsions (122,123). Exacerbation of withdrawal by NE is consistent with the hypothesis of French and Palmer (82), who suggest that withdrawal from ethanol is mediated, to a large extent, through supersensitive central NE receptors.

In light of these findings it appears that ethanol withdrawal severity is directly related to NE and inversely to dopamine release. That is, NE exacerbates withdrawal while dopamine ameliorates it. Withdrawal reflects the release of NE, the severity being determined by amount released, and/or the state of the NE-receptors (i.e. supersensitive). This hypothesis further suggests, however, that dopamine, or dopamine agonist, may be useful in reducing the severity of ethanol withdrawal.

These results reported for ethanol seem to be compatible with the assumption that long lasting depletion of brain CA's is compensated for by induction of neuronal supersensitivity for NE and DPA. The recent finding by Engel and Liljequist (124) that long-term ethanol treatment resulted in an enhanced sensitivity of DPA receptors in the nucleus accumbens supports this hypothesis.

Blasig et al. (92) similarly proposed that neuronal supersensitivity for CA's is an important factor in considering the mechanisms involved in expression of morphine abstinence. Evidence for this hypothesis is based on the work of Way (91), Maruyama and Takemori (125) and Herz et al. (126). In the work by Maruyama and Takemori (129) the authors



conclude that "the full expression of abstinence syndrome in morphine-dependent mice appears to require the integrity of the central stores of NE and DPA, especially the latter amine". In contrast, Herz et al. provide evidence which supports the notion, that noradrenergic mechanisms are involved in the expression of withdrawal, while the role of DA is not clear. The authors conclude that contradictory results concerning the role of biogenic amines in morphine abstinence may be due to the type of symptom studied. They suggest that brain CA protection against excessive abstinence signs (127) should be restricted to convulsive activity rather than predicting a generalized effect.

5-HT. Goldstein (116) reported that drugs aimed at serotonin had no effect on the withdrawal reaction induced by ethanol. Mice treated with PCPA to block 5-HT synthesis or with tryptophan to increase brain levels of 5-HT showed the same withdrawal scores as controls.

Utilizing a different withdrawal sign Collier et al. (107) found that drugs that are 5-HT antagonists, namely, methysergide, methergolin and MA 1420 given one hour prior to head twitch counting in ethanol dependent mice significantly reduced the incidence of this withdrawal symptom, while administration of 5-HTP fifteen minutes prior to counting, significantly increased the incidence of this sign (107). Other reports indicate that there are inconsistent effects with intracerebral administration of 5-HT during the withdrawal phase as evidenced by increases, decreases or no effect on withdrawal scores of ethanol dependent mice (99). Only a slight increase in the withdrawal reaction was obtained with 5-HTP administration, and PCPA was without any significant effects. However, the same report (99) showing exacerbation of withdrawal convulsions rather than amelioration as reported by Collier et al. (107), of withdrawal convulsions following intracerebral injections of methysergide suggests that 5-HT may modulate ethanol-induced withdrawal. Differences between these findings and those of Collier may reflect differences in the abstinence sign measured, and the time of assessing the withdrawal sign. Griffiths et al. (37) found that administration of PCPA to mice prevents the rise in brain 5-HT concentration associated with ethanol withdrawal induced by inhalation but does not affect the increase in brain CA's which occurs at the same time. The locomotor, excitement, pilo-erection, tremor and handling convulsions which occur during ethanol withdrawal were not affected. These results suggest that the increase in brain 5-HT which occurs in ethanol withdrawal is a consequence of increased 5-HT synthesis and that it is probably not involved in the above behavioral changes associated with the early phase of ethanol withdrawal (up to twenty-four hours). The assessment of effects of drugs

which modify 5-HT on the withdrawal reaction occurred twenty-four hours after the last dose of ethanol in Collier's laboratory compared to a much earlier assessment of between one and twenty hours in other laboratories. This may suggest that 5-HT may mediate behavioral changes of ethanol withdrawal during the latter phase and possibly CA's may mediate the early phase of withdrawal.

Investigators have made several attempts to link morphine with 5-HT during the withdrawal states (88,128). Shen et al. (50) proposed that 5-HT may be associated with morphine abstinence since PCPA and 5,6-dihydroxytryptamine inhibited wet-dog shakes following naloxone administration to tolerant-dependent morphine animals. Furthermore, Way et al. (129) and Shen et al. (50) reported that PCPA, which inhibits tryptophan-5-hydroxylase and thereby the synthesis of 5-HT, antagonized withdrawal symptomatology in mice. Although Way et al. (129) have shown that chronic administration of morphine increases the rate of brain 5-HT synthesis, no change in brain 5-HT synthesis was found in mice showing a definite withdrawal syndrome (129). Thus, it is possible that the increase in brain 5-HT synthesis during the tolerant-dependent state is not a necessary condition for the development of withdrawal symptomatology.

**ACH.** Evidence for a possible direct cholinergic involvement in ethanol preference has been presented by Ho and Kissin (130), but there are no reports correlating other behavioral measures with effects of ethanol on ACH release (131) and acetylcholinesterase activity (132). In addition, ethanol had little or no effect on the toxicity of the anticholinesterase agent, parathion (133).

With regard to ethanol withdrawal, Goldstein (116) found that mice treated with physostigmine, atropine or dihydro- $\beta$ -erythroidine (a curare-like cholinergic blocker that acts centrally) showed the same withdrawal scores as controls. In contrast, utilizing a different sign of ethanol withdrawal, Collier et al. (107) found that intracerebroventricular injection of ACH or carbachol increased the incidence of ethanol withdrawal head twitching whereas nicotine reduced it. By the intraperitoneal route, physostigmine increased head twitching, while the muscarinic blocking agent, hyoscine, lessened its incidence. Similarly, the effect of drugs that alter cholinergic function on opiate withdrawal is not conclusive. Grumbach (134) reported that atropine increased, and physostigmine decreased, abstinence sign in rats. However, Crosslands (135) found opposite effects in dependent rats, that is, atropine lessened and physostigmine worsened the withdrawal syndrome induced by naloxone.

These conflicting results concerning the role of ACH in both ethanol withdrawal and opiate withdrawal prevents any

conclusion concerning the function of ACH in withdrawal states induced by removal of either of these agents.

**GABA.** The effect of drugs that affect GABAminergic pathways on ethanol withdrawal has been investigated (116). Goldstein (116,136) showed that GABA appears to counteract withdrawal hyperexcitability in ethanol-dependent mice. The convulsion scores were reduced by aminooxyacetic acid, an inhibitor of GABA transaminase that is known to increase brain levels of GABA (136,137), whereas picrotoxin, a GABA antagonist (137) produced a brief but sharp increase in the scores. Goldstein (116) points out that these results suggest that GABA is an effective endogenous anticonvulsant. The possibility that the GABA system is functionally upset during alcohol withdrawal is supported by the finding that brain levels of GABA are lower than normal during the alcohol withdrawal reaction in mice treated with the Goldstein-Pal (138) inhalation method.

Except for the work of Ho et al. (51) showing enhancement of tolerance and physical dependence with drugs affecting GABAminergic activity and bicuculline, a GABA antagonist inhibiting tolerance and physical dependence, no other research has been accomplished with respect to the interaction of GABA and the chronic effects of opiates. It appears that increases in GABA tend to enhance the development of tolerance and physical dependence whereas decreases in GABA activity reduce naloxone precipitated jumping in dependent animals exposed to opiates.

**Adenosine 3'5'-cyclic Monophosphate (cAMP).** Several investigators have attempted to correlate acute as well as chronic actions of ethanol with levels of cAMP (74,76,79-81), but much less is known about the role of cAMP in ethanol induced withdrawal. Recently, Collier et al. (107) studied the effects of drugs affecting endogenous cyclic nucleotides on ethanol withdrawal head twitches in mice. By the intracerebroventricular route, db cyclic AMP lessened the incidence of withdrawal head twitches whereas db cyclic GMP increased the incidence. Adenosine triphosphate (ATP) and guanosine triphosphate (GTP) affected the incidence of twitches in the same direction as did the dibutyryl salts of the corresponding cyclic nucleotides. The phosphodiesterase inhibitors, theophylline and 3-isobutyl-1-methyl xanthine (IBMX), increased withdrawal head twitching, but imidazole, a phosphodiesterase stimulant, was inactive. Prostaglandins E1 and E2, which increase brain cAMP (139), also increased head twitching but prostaglandin F2, which has little effect on brain cAMP was inactive. Collier et al. (107) suggest that drugs which increase the level of cGMP increase head twitching and drugs which increase the level of cAMP inhibit head twitching. Hence, head twitching may arise from an increase in the ratio of cGMP:cAMP, perhaps the result of a change in the balance of

neurohumoral mediators, particularly catecholamines. No similar reports have been noted for opiates.

#### IONS AND OPIATES AND ETHANOL

The acute and chronic effects of opiates have been well documented (Chapter 15). Kakunago et al. (140) found that  $\text{Ca}^{++}$ , but not other ions, antagonized opiate induced analgesia. Shikimi et al. (141) suggested that the analgesic action of morphine may be due to the opiates' effect on  $\text{Ca}^{++}$  flux. The same researchers (141,142) demonstrated that morphine decreased whole brain  $\text{Ca}^{++}$  in mice, an effect to which tolerance developed. Ross and his colleagues have extended the work of Shikimi et al. (141) by demonstrating that opiate agonist cause dose-dependent decreases of  $\text{Ca}^{++}$  in regional areas of the brain, and that this depletion can be inhibited by naloxone (143).

Although there are few definitive reports concerning the role of  $\text{Ca}^{++}$  in acute and chronic effects of ethanol, Ross et al. (143) reported that morphine and ethanol deplete  $\text{Ca}^{++}$  in the same regional areas of the brain. This effect is selectively antagonized by the stereospecific narcotic antagonist, naloxone. Furthermore, cross-tolerance between morphine and ethanol with reference to their ability to deplete  $\text{Ca}^{++}$  has been demonstrated (144). The work by Ross et al. (144) suggested that ethanol and opiates have in common at least one biochemical mechanism. In this regard, it is known that administration of  $\text{Ca}^{++}$  antagonizes the tolerance of development to the analgesic effect of morphine. As well, Sangri and Gershon (145) reported that  $\text{Ca}^{++}$  antagonized the development of dependence to opiates, while chronic administration of  $\text{Ca}^{++}$  during morphine exposure to mice significantly inhibited naloxone-induced jumping. As well, it has been found that calcium gluconate administered daily to mice undergoing ethanol vapor exposure significantly reduced the resultant withdrawal syndrome upon removal from the ethanol vapor chambers (146). In addition, naloxone was also found to block ethanol-induced depletion of  $\text{Ca}^{++}$  content in the brain of mice exposed to ethanol vapor (17).

#### CONCLUSION

As with the effects of administration of opiates or ethanol on endogenous neurochemical mechanisms, the effects of prior manipulation of these neurochemicals on subsequent tolerance to, dependence on, and withdrawal from these agents is similarly confusing.

It is apparent from the evidence presented that there is still considerable controversy surrounding the neurochemical

effects and determinants of both opiates and ethanol. A definitive case cannot yet be made for any single biogenic amine, ion, or other endogenous substance as the initiator of dependence producing properties of either agent.

There are certain similarities between opiates and ethanol that became evident on inspection of the results presented in the previous sections and summarized in Table 1.

There are also some distinct differences that are evident in some reports. For instance, while dopamine has been reported to ameliorate ethanol-induced withdrawal (120), exacerbation of morphine withdrawal has been suggested to occur with stimulation of dopamine receptors (102). However, this exacerbation of withdrawal pertained primarily to "dominant" withdrawal signs (e.g. jumping) while "recessive" signs (e.g. diarrhea) decreased (126).

Thus, the definition of exacerbation or amelioration depends on the importance ascribed to particular withdrawal signs, and that a conclusion concerning the effects of a drug on abstinence from either opiates or ethanol rests on this definition.

#### TETRAHYDROISOQUINOLINES: A POSSIBLE LINK BETWEEN OPIATES AND ETHANOL

The similarities between opiates and ethanol summarized in Table 1 on behavioral parameters, alteration of endogenous neurochemical function and the effects of endogenous neurochemical function and the effects of prior modification of monoamines on dependence and withdrawal, suggest that there may exist a "link" between these two classes of addictive drugs. A possible biochemical rationale for this link was provided in 1970, when two laboratories simultaneously published articles reporting the formation of simple and complex tetrahydroisoquinolines (TIQ) alkaloids as a consequence of ethanol metabolism (147,148). As described earlier (see earlier chapters), these substances are formed by the spontaneous condensation of an aldehyde with an appropriate  $\beta$ -arylalkylamine (148,149). This discovery was significant in two aspects: It was the first demonstration that mammalian tissues could "synthesize" alkaloids, and benzyloisoquinoline alkaloids (e.g. tetrahydropapaveroline, THP) are requisite intermediates in the biosynthesis of morphine in the poppy plant *Papaver somniferum* (149,151). The recent demonstration of the endogenous formations of TIQ alkaloids during ethanol intoxication (152) and identification by Sandler and co-workers (153) of urinary excretion of two dopamine-derived TIQ's (salsolinol and tetrahydropapaveroline) in Parkinson patients receiving L-dopa and ethanol supports the hypothesis that endogenous alkaloid formation could represent important metabolic sequelae of

TABLE 1

## Opiate-Ethanol Interactions

Behavioral Interactions of Opiates and Ethanol	Effect of opiates and ethanol on endogenous neurochemical mechanisms	Effect of neurochemical manipulation on the actions of opiates and ethanol
Blockade of ethanol narcosis by naloxone (18)	Increased NE turnover following chronic alcohol or morphine administration (24,29-34)	Decreased tolerance (T) and physical dependence (PD) by cycloheximide (84-87)
Increased tolerance to morphine following ethanol (144)	Increased brain ACh concentration by acute morphine or ethanol (64-66,69,70)	Decreased T and PD by propranolol (88,95)
Increased VAC following naloxone or naltrexone (13,14)	Naloxone-sensitive depletion of brain $Ca^{++}$ by acute morphine or ethanol (143,144)	Exacerbation of the withdrawal syndrome (WS) by NE-agonists (89,95)
Decreased VAC following morphine (11,12)	Increased adenylate cyclase activity following chronic ethanol or morphine (80,81) and during withdrawal (83,92)	$Ca^{++}$ replacement attenuates physical dependence (145,146)
Decreased ethanol withdrawal severity by morphine (19)		
Inhibition of ethanol dependence by naloxone (17)	Opiate receptor affinity for opiates, ethanol and salsolinol (161-163,168)	

acute and chronic ethanol intoxication. These findings formed the basis for an intriguing, though controversial hypothesis, linking the opiates to ethanol. Such a theory was proposed by Davis, and her colleagues (148) centering on the formation of tetrahydropapaveroline (THP, norlaudnosoline), the adduct of dopamine and its aldehyde (3,4-dihydroxy-phenylacetaldehyde). The biogenesis of this alkaloid in mammalian tissues suggested that common biochemical mechanisms may exist between opiates and ethanol.

Cohen and Collins (147) proposed an alternate hypothesis, suggesting that simple TIQ's could contribute to the acute and chronic effects of ethanol intoxication by interfering with adrenergic function (c.f. Chapter 8). As norepinephrine (NE) and dopamine (DPA) constitute the major catecholamines in the CNS, it is entirely possible the TIQ derivatives formed *in vivo* from these neuroamine following ethanol consumption, could contribute to the pharmacological effects of ethanol by interfering with catecholaminergic mechanisms in the CNS and in the periphery. These alkaloids have been shown by Cohen and his group (154-156) to have some properties in common with the catecholamines. They compete for the same uptake and storage mechanisms and possibly act as false transmitters. Under certain experimental conditions, they are NE-like in action and are secreted in a similar manner as NE (18).

Since 1970, there has been considerable effort devoted to determining the pharmacological activity of these compounds, and also proving their formation *in vivo* following ethanol consumption. Sandler et al. (153) and Collins and Bigdeli have identified that TIQ's are present *in vivo* after ethanol, but in both cases metabolic precursors (i.e. L-dopa) or other drugs were also present. In fact, there are reports that without such pretreatment, TIQ's cannot be identified from brains of ethanol-intoxicated animals (157). The inability to find TIQ's after ethanol alone may mean that current techniques are not yet sensitive enough. If this is found to be the case, then the absolute concentration of TIQ's in the CNS following ethanol will be quite small. However, if this small amount is localized in specific nerve endings, it may be sufficient to induce significant metabolic and functional changes during the course of chronic ethanol intoxication.

There has been much more success in defining the pharmacological activity of TIQ alkaloids (c.f. Chapters 8 and 10). This section will focus on the simple TIQ's derived from dopamine and L-dopa, salsolinol and 3-carboxysalsolinol, rather than the complex benzyl-TIQ's and their derivatives for the following reasons: i) the formation of the former requires the ethanol metabolite, acetaldehyde, while the latter does not, and ii) kinetically, the formation of the former two alkaloids is favored (158).

How these alkaloids might serve to link the biochemical and behavioral actions of ethanol and opiates is speculative, but enough is known of their pharmacology to suggest some possible mechanisms.

The chronic administration of ethanol and opiates produces an increase in central NE. The mechanism of this metabolic consequence is not known for either class of drug, but the formation of TIQ alkaloids may provide an explanation for the increase following ethanol. The formation of dopamine-derived TIQ's would decrease the synthesis of NE by removing the substrate for dopamine- $\beta$ -hydroxylase. This decrease of NE would remove end-product inhibition and stimulate increase synthesis (turnover). Furthermore, salsolinol is known to release NE from nerve endings and inhibit its re-uptake (154-156). This would additionally decrease end-product inhibition of tyrosine hydroxylase and promote increased NE-turnover.

It is noteworthy that acute administration of salsolinol to mice undergoing withdrawal from ethanol vapor was found (depending upon the dose) to either ameliorate (low dose) or exacerbate ethanol induced withdrawal symptoms (97,101). Salsolinol as previously discussed is known to release neural stores of catecholamines (155) so the observed exacerbation could be the result of NE release and thus activation of central NE- $\alpha$ -receptors. Implicit in this argument is the fact that NE- $\alpha$ -activation supercedes DPA- $\alpha$ -activation because these alkaloids release both DPA and NE from neural stores (155). The biphasic action of salsolinol, although appearing at first inspection to be contradictory, may be explained by the recent observations that salsolinol can block  $\alpha$ -receptors (159) and is an agonist at dopamine receptors (160,161). Thus, large exogenous doses of salsolinol probably exert their effect through a catecholamine release, while the smaller amount acts through direct receptor antagonism ( $\alpha$ ) and/or activation (DPA).

The finding that morphine attenuates the ethanol withdrawal syndrome (19), and that naloxone inhibits the development of dependence to ethanol (17) suggests that ethanol, or some metabolite, affects the opiate receptor. It was recently reported that salsolinol behaves as a partial opiate agonist in the guinea pig ileum (162). Additionally, salsolinol depletes regional brain  $Ca^{++}$ , as does morphine, when administered peripherally (143). Both of the above effects of salsolinol are inhibited by naloxone (143,162), suggesting that the site of action is the opiate receptor. While these findings are highly suggestive, much more work must be accomplished before definite conclusions can be drawn.

There are further indications that salsolinol and 3-carboxysalsolinol may be important in the actions of ethanol and



may interact with the opiate receptor (163,164). Salsolinol was found to stereospecifically bind to the opiate receptor in guinea pig brain with a potency of  $1 \times 10^{-3}$  that of normorphine (163). Both of these alkaloids prolong ethanol-induced narcosis (see Chapter 10) but have no effect on barbiturate induced hypnosis (165). As well, 3-carboxysalsolinol has recently been shown to produce analgesia by itself, and to potentiate morphine analgesia. Again, both of these effects are inhibited by naloxone (166). Intracerebral administration of salsolinol has also been found to affect morphine analgesia in mice (167). These results tend to support a partial agonist role for salsolinol with regard to opiate receptor interaction.

These speculations, while intriguing, require the demonstration that TIQ alkaloids are formed *in vivo* during ethanol intoxication, and that there be formation of significant amounts of these alkaloids to produce physical dependence. The term "significant" is relative in this application, because, as mentioned earlier, localized formation of these compounds in synaptic nerve endings could provide physiologically active concentrations which might be below the detectable limit for current techniques.

#### SUMMARY

Although a tremendous amount of scientific inquiry has been directed at defining the mechanisms responsible for the behavioral and biochemical alterations induced by ethanol and opiates, it is evident that considerable controversy exists in virtually all areas of research. There are several reasons for this, as discussed in this review, and it appears clear that these areas of controversy will not be resolved for some time. Perhaps a major cause of the differences is a lack of agreement concerning methods for induction of dependence and most important, its subsequent measurement. What constitutes a withdrawal sign, when does withdrawal begin and end, what is the relative importance of each sign, and what is the best way to objectively measure (quantify) each sign? All are areas of considerable disagreement.

Nevertheless, in our opinion, there is sufficient evidence in the literature to suggest that ethanol and opiates have biochemical and behavioral mechanisms in common, and that this hypothesis warrants further investigation.

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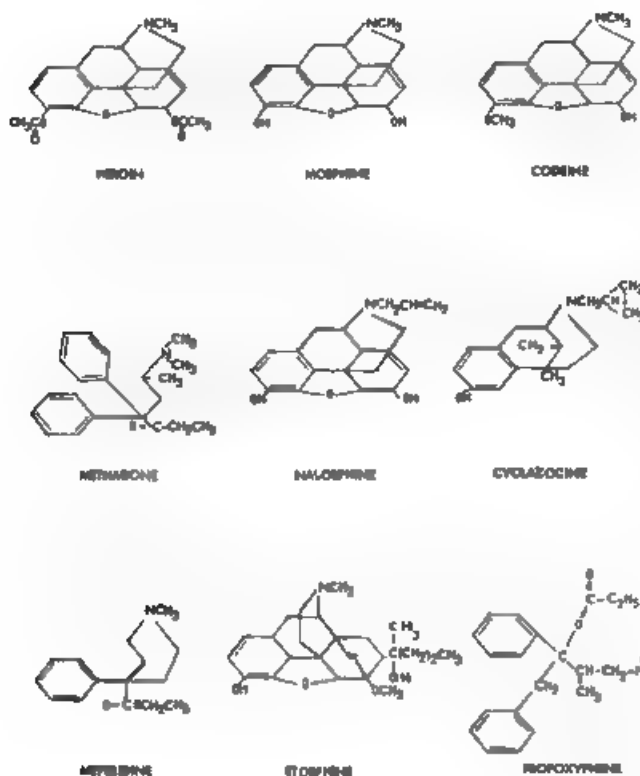
### 13. NEUROCHEMICAL ASPECTS OF OPIATE DEPENDENCE: AN OVERVIEW

Doris H. Clouet

New York State Office of Drug Abuse Services, Testing and  
Research Laboratory, Brooklyn, New York, 11217.

#### INTRODUCTION

Narcotic analgesic drugs, or the opiates (more properly the opioids), have varied chemical and pharmacological characteristics, ranging chemically from the simple meperidine molecule to the structurally complex etorphine molecule (Figure 1), and ranging behaviorally from the euphoriant heroin to the antidiarrheal agent, diphenoxylate. All opiates possess, however, the characteristics that define the drug class: the ability to induce tolerance and physical and psychic dependence with chronic drug use (1).



*Fig. 1. Typical  
Opiate Structures.  
Chemical structures  
of some narcotic  
analgesic agonists  
and antagonists  
(nalorphine and  
cyclazocine).*

The neurochemical mechanisms underlying the phenomena of tolerance, abstinence and drug-seeking behavior have been explored in depth, especially during the past ten years. In this overview, I shall describe briefly the results of some of the studies and the conclusions that may be reached.

#### *SITES OF OPIATE ACTION IN THE NERVOUS SYSTEM*

##### Anatomical Sites

Opiates produce responses in most brain areas, in the pituitary gland, ganglion cells and spinal cord, as well as at some neuronal junctions outside the central nervous system (2). Opiates depress impulse transmission only at a few neuronal junctions in the autonomic nervous system; these sites are not characteristic of species, organ or tissue (3). Impulse flow in cholinergic junctions in the guinea-pig ileum and rat vagus, in adrenergic neuronal junctions in the cat nictitating membrane and mouse vas deferens, is inhibited by morphine and other opiates (3). In most mammalian species opiates increase the contractile activity of the intestine, possibly via a serotonergic neuron (4). In the spinal cord, a direct action of opiates is demonstrated in experiments in which the drugs are shown to alter spinal reflexes equally well in intact and in spinally-transsected animals (5). There are, on the other hand, other effects of morphine on spinal neurons involving afferent transmission that require an intact cord (6). In the pons-medulla, where vegetative functions such as respiration, regulation of blood pressure and emesis are sited, opiates act to disturb these functions (2). Temperature regulation is affected by the intrahypothalamic application of opiates, and the hypophyseal-hypothalamic hormonal system is also affected by opiate administration (7). The limbic system including the neocortex has also been implicated in opiate actions, particularly behavioral responses (8). Thus, the sites of opiate action are located throughout the central nervous system and at some peripheral neuronal junctions.

##### Neurochemical Sites

When radiolabelled opiates are administered to laboratory animals, the drugs are found in all brain regions with a temporal and spatial distribution related to the lipid solubilities of the drugs rather than to their pharmacological potencies (9). However, the potency of one opiate, etorphine, is so high that an effective dose via the intracerebral route is about 1/100th that of morphine; a dose that diminishes non-specific binding so that specific changes may be measured. The binding of  $^3\text{H}$ -etorphine in synaptic membranes of rat brain is

reduced to one-half when naloxone is also injected in doses sufficient to block the pharmacological responses of etorphine (10).

Opiates bind to brain tissue *in vitro* also, in an interaction that is stereospecific, with high affinity of drug to receptor and a saturable binding (11-13). Isolated synaptic membranes from many brain areas contain opiate receptors, with a heterogeneous distribution (14,15). Thus, synaptic membranes are identified as the site of the initial drug:receptor interaction.

### Molecular Sites

The stereospecific binding of opiates to their receptors is competitive, with  $EC_{50}$  related to the potencies of the drugs (16). The binding of agonists and antagonists can be distinguished by sodium ions, which decrease agonist binding and enhance antagonist binding (17). The ratios of  $EC_{50}$  values determined in the presence and absence of 100 mM NaCl range from 1.0 for naloxone and naltrexone (the purest antagonists) to 12-60 for agonists, with mixed agonist-antagonists in the intermediate range (16).

The opiate receptor has not been isolated as yet. However, an opiate:receptor complex has been solubilized from a rat brain synaptosomal preparation with the non-ionic detergent, Brij 36T (18). The bound opiate is dissociated by treatment with proteolytic enzymes, sulfhydryl reagents and heat, suggesting that the receptor is a protein (18).

After the initial drug:receptor complex is formed, it must produce a transduction of the information to secondary effector processes. A tentative identification of the cyclic nucleotide systems as the mediators of this transduction is supported by two general lines of evidence: (1) the nature of the cAMP and cGMP systems as amplifiers in the regulation of neurotransmission by ions, neurotransmitters and neurohormones (19), and (2) the changes found experimentally in the levels of cyclic nucleotides and the sensitivity of cyclases in brain (20-22) and in neuronal cells in culture (23,24). The nature of the further reactions in the neuron, and elsewhere, is related to the specific functions of the cells involved.

The development of tolerance may be considered as a series of adaptive responses to the continual presence of a potent drug. Since tolerance had been demonstrated in many neurochemical parameters, I shall discuss those for which a biochemical mechanism is known in appropriate sections of this chapter.

### *ROLE OF NEUROTRANSMITTERS IN OPIATE ACTION AND DEPENDENCE*



### Catecholamines

The administration of morphine or other opiates to laboratory animals induces alterations in the content and the rate of turnover of catecholamines in brain. Both dopamine and norepinephrine levels fall after opiates are administered acutely (25). The effect is dose-dependent and multiphasic (26,27). The most prominent effect of opiates on the turnover of catecholamines is a sharp rise in the rate of dopamine biosynthesis in striatum after a single dose of morphine (29,30). Tolerance develops in this biochemical parameter as opiates are used chronically (31).

The administration of non-opiate drugs to animals often affects the response to opiates. Drugs that tend to increase the levels of dopamine at the receptor (such as iproniazid, apomorphine or cocaine) generally enhance the pharmacological responses to opiates, while drugs that decrease dopamine levels (such as haloperidol, alpha-methylDOPA) inhibit opiate action (32).

### Acetylcholine

Opiates have an antirelease action at cholinergic synapses, and effect antagonized by naloxone and absent in tolerant animals (33). The antirelease activity has been demonstrated in cortical slices, in perfused brain and on the cortical surface, as well as *in vivo* (34). In morphine-tolerant animals, the dose-effect curve for inhibition of neocortical acetylcholine release is shifted to the right, *i.e.* tolerance develops in this response (35). During withdrawal from morphine use, brain acetylcholine is utilized at an enhanced rate (36), and an increase in the level of free acetylcholine is found, suggesting that release is enhanced after drug removal (37).

In low concentrations, opiates prevent contraction of stimulated guinea-pig ileum preparations by inhibiting acetylcholine release (38). Short term tolerance to this effect has been attributed to a disuse supersensitivity phenomenon: after exposure to an opiate for a period of time, the ileum increases its response to a fixed dose of acetylcholine three to five-fold (39).

### Serotonin

The acute administration of morphine to rodents accelerates the rate of biosynthesis of serotonin in brain (40). This effect is located in the forebrain (41). The biochemical correlate for this effect may be changes in tryptophan hydroxylase activity in nerve-ending fractions of forebrain areas;

the synaptosomal enzyme activity decreases two or three hours after initial dose of morphine, and is increased in tolerant animals (42). An inhibitor of serotonin biosynthesis, *p*-chlorophenylalanine, antagonizes the pharmacological responses to morphine (43-45). The ratio of serotonin to catecholamines in brain seems to be more important than actual levels of the biogenic amines for morphine-induced analgesia (46), temperature alterations (47,48) or effects on oxytremorine analgesia (49). In these studies, norepinephrine seems to be a morphine antagonist, while serotonin potentiates morphine responses.

The stimulation of contraction of the dog intestine by morphine occurs at the same time that endogenous serotonin is released (4), an effect antagonized by naloxone (50). Tolerance develops in this biochemical parameter in tolerant animals (50).

#### Other Neurotransmitters

GABA levels seem to be important for the expression of some pharmacological responses to morphine administration. When GABA levels in brain are increased either by administering GABA, or an inhibitor of GABA catabolism, aminooxyacetic acid, the responses to acute morphine are reduced (51). The development of tolerance and dependence are accelerated by these same agents (51). In subcortical areas of brain of morphine-tolerant rats, the levels of GABA are increased. Thus, GABA acts as an opiate antagonist (in the pharmacological sense of the word).

Histamine release in peripheral tissues is stimulated by morphine (52). In the CNS, however, no changes in histamine levels have been seen in animals treated acutely with morphine. In morphine-tolerant animals, however, hypothalamic levels of histamine are reduced, and are even lower in the hypothalamus of animals undergoing withdrawal (53).

#### Unique Role for One Neurotransmitter

The studies just described suggest that all known neurotransmitters are involved in the actions of opiates (and in the expression of these actions), and that no one neurotransmitter plays a unique role in either opiate action or dependence.

#### ROLE OF NEUROREGULATORS IN OPIATE ACTIONS AND DEPENDENCE

##### Ions

Any disturbance of the ionic milieu of the neuronal cell can be expected to alter neuronal function. However, calcium

ions seem to have a specific role in the action of opiates. The analgesic effect of morphine in mice is depressed by the intracisternal administration of calcium ions, and potentiated by the administration of a calcium ion-chelator, EDTA (54). Brain levels of this ion are lower in mice treated with morphine, an effect to which tolerance develops (55). In rats, a depletion of calcium ions is produced by morphine administration (56,57). Not only calcium ions, but also magnesium ions and manganese ions increase the  $AD_{50}$  for morphine (58). These latter investigators have concluded the pharmacological responses to morphine in mice are directly related to the intracellular levels of calcium ions in brain (58). Lanthanum ions, which are antagonists of calcium ions in several biological systems, produce analgesia when introduced into the CSF, an effect antagonized by calcium ions or naloxone (59).

The monovalent ion, lithium has a sedative effect in man and animals. In mice, morphine is able to reverse the inhibition of spontaneous locomotor activity induced by lithium ions (60). Conversely, lithium ions reverse some morphine responses; namely, analgesia (61) and body temperature regulation (62).

### Prostaglandins

In the guinea-pig ileum, morphine inhibits the contractions evoked by prostaglandins (PG) as well as by electrical stimulation (63). In rat brain homogenates, opiates inhibit the stimulation of cAMP formation by PGs (64). Similar effects have been observed in cultured hybrid cells (neuroblastoma X glioma) (23,24). At low concentrations of opiate, the responsiveness of adenylate cyclase to PGs is enhanced, while at higher concentrations, the enzyme is inhibited (23). Since PGs hyperpolarized neuronal cells (65), thus inhibiting the transmission of the nerve impulse, a reversal of PG-induced hyperpolarization by opiates would lead to the activation of inhibitory pathways, and a selective depression of neuronal activity.

### Cyclic Nucleotides

When cAMP is injected systemically into rodents together with morphine, the analgesic response is antagonized (66). In morphine-tolerant animals, the administration of cAMP systemically (67) or into the CSF (68) increases withdrawal signs. Adenine, adenosine and ATP also decrease the acute effects of opiates (69), suggesting that the adenosine moiety may be responsible for the effects.

Opiate administration to animals has varying effects on the levels of cyclic nucleotides and the activity of adenylate

and guanylate cyclases (70). Both the nucleotide levels and the cyclase activities may be expected to fluctuate widely over a short time period in whole tissue, making it difficult to discern relevant alterations in either parameter. In hybrid cells in culture, however, opiates can be shown to have effects on the cyclic nucleotide system. The stimulation of adenylyate cyclase activity by PGEs is inhibited by morphine (71). An effect that is stereospecific is the increase in cGMP levels induced by morphine in these cells (65). In rat brain, synaptosomal adenylyate cyclase activity is not affected by opiates added in vitro (21). Dopamine-sensitive adenylyate cyclase in synaptosomal preparations from rat striatum is not inhibited by opiates (20). Thus, opiates do not act directly on postsynaptic dopamine receptors in this brain region, as neuroleptics do.

### Peptides

Responses to morphine are altered by the simultaneous administration of neuroactive peptides. For example: an analog of vasopressin increases the analgesic effect of morphine in mice (72) and somatostatin antagonizes the stimulation of growth hormone (GH) release induced by morphine (73).  $\beta$ -melanotropin and adrenocorticotropin (ACTH)<sub>1-24</sub> antagonize the pharmacological responses to morphine (74) and substance P abolishes the abstinence syndrome (75). The injection of thyrotropin-releasing hormone (TRH) into certain brain areas produces shaking behavior in rats very like the abstinent shaking produced by the micro-injection of naloxone into the same areas in morphine-tolerant animals (76).

It may be necessary to re-examine some of these effects in light of the possibility that the peptide preparations may have been contaminated with the endogenous morphine-like peptides recently discovered.

### Pituitary Peptide Hormones

The administration of narcotic analgesic drugs to man or animals produces changes in the rates of release of peptide hormones from the anterior and posterior gland. Acute administration of morphine increases the secretion of ACTH from the pituitary: this is a relatively specific effect since it is blocked by nalorphine, and induced only with the L-isomer of methadone (77). Chronic morphine treatment reduces ACTH release to a low normal level in man (78) and in animals (79). Low doses of morphine increase the secretion of GH, while higher doses increase the secretion of both GH and gonadotropins (80). In contrast, morphine inhibits the release of TSH (81). The relationship of the hypothalamus and other

brain areas to the pituitary gland is complex (Figure 2). Neuronal cells in discrete hypothalamic nuclei contain the oligopeptide releasing factors that are transported to the pituitary where they influence the rate of tropic hormone

#### PITUITARY - HYPOTHALAMUS CYCLE

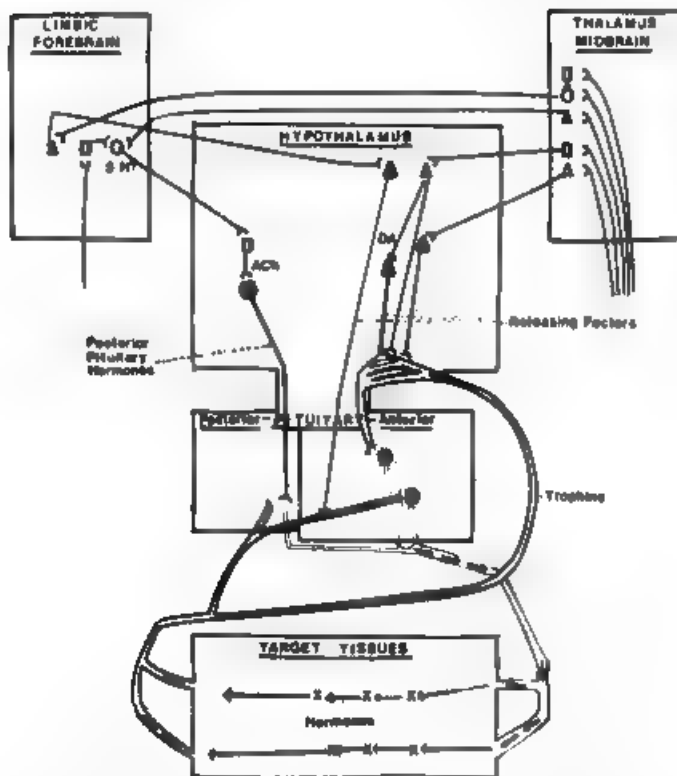


Fig. 2. The interrelations of the pituitary gland, the hypothalamus, 'target tissues' and other brain areas is shown in this figure.

release. For some of the effects of opiates on the release of peptide hormones from the anterior pituitary, the hypothalamic dopaminergic neurons are the mediators. The neurons controlling LH-RF, GH-IF, prolactin-inhibitory factor and MIF are all dopaminergic and inhibited by morphine and methadone (82), thus the release of LH is inhibited while the release of the other peptide hormones is accelerated by the inhibition of inhibitory factors.

Morphine produces an antidiurectic response that is effected in part by the release of antidiuretic hormone from the posterior pituitary gland (83). With the development of tolerance, the antidiurectic effect disappears and a diuretic effect may sometimes be found (84). The peptide hormones of the posterior pituitary gland, vasopressin and oxytocin, are synthesized in the supraoptic and paraventricular nuclei of the hypothalamus, presumably from a larger peptide (85). It is possible that the effects of opiates on these hormones is also mediated by neurotransmitters in the hypothalamus.

The regulation of synthesis and release of hypophyseal peptide hormones by neural pathways and by feedback control by pituitary hormones, hormones in the target tissue and hypothalamic releasing factors is suggested in Figure 2. An additional pathway for the regulation is the short feedback loop whereby pituitary hormones enter the CSF in the basilar cisterns and are transported to the periventricular organ system of the hypothalamus, and to other brain areas, and hypothalamic releasing hormones are released into the CSF to return directly to the site of their origin (86,87). These routes may be important for the dissemination of the morphine-like peptides.

#### Unique Role for a Neuroregulatory System

The studies described above suggest that all neuroregulatory systems play a role in the actions of opiates, and that neuronal responses to the presence of opiates are mediated through normal cell mechanisms.

#### *ENDORPHINS*

The presence in nervous tissue of endogenous ligands, substances that bind stereospecifically to opiate receptors, was suggested by the unique characteristics of the receptors. The discovery of possible ligand activity was first reported by Terenius and Wahlstrom (88) and by Hughes and his colleagues (89). Other groups have described the partial purification of 'morphine-like' factors from brain (90) and from the pituitary gland (91). The chemical identification of brain 'morphine-like' factor as two pentapeptides with the following structure:  $\text{tyr-gly-gly-phe-X}$ , with X=methionine or leucine, and the recognition that the met-peptide is a sequence of the pituitary peptide hormone,  $\beta$ -lipotropin, was made by Hughes, Kosterlitz and their colleagues (92). These pentapeptides are called enkephalins. However, the generic name for the enkephalins and other substances with morphine-like activity is endorphin. Segments of  $\beta$ -lipotropin containing the met-enkephalin sequence of amino acids bind stereospecifically to opiate receptors, and act like opiates in isolated tissue preparations, guinea-pig ileum and mouse vas deferens (93,94). Some of the endorphins are shown in Figure 3. Morphine-like pharmacological activity *in vivo* has also been demonstrated for the endorphins when the destruction of the peptides by tissue esterases is prevented (95). Opiate-like effects, antagonism by naloxone, tolerance and cross-tolerance have all been demonstrated for the endorphins (96-98). Thus, these peptides are endogenous substances with all of the specific biological activity

of the narcotic analgesic drugs.

Endorphins are distributed throughout the central nervous system (99). The regulation of their biosynthesis and catabolism, and the sites of their biosynthesis and

#### ENDORPHINS

Tyr-Gly-Gly-Phe-Leu	Leu-enkephalin
-----	-----
61                      65	
Tyr-Gly-Gly-Phe-Met	Met-enkephalin
-----	-----
66                      69	
Tyr-Gly-Gly-Phe-Met-Thr-Ser-Gly-Lys	$\beta$ -LPH (61-69)
-----	-----
Tyr-Gly-Gly-Phe-Met-Thr-Ser-Gly-Lys-	
71                      76	$\alpha$ -endorphin
Ser-Gln-Thr-Pro-Leu-Val-Thr	
-----	-----
Tyr-Gly-Gly-Phe-Met-Thr-Ser-Gly-Lys-	
71	
Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-	$\beta$ -LPH (61-91)
80    81	( $\beta$ -endorphin)
Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His-	
91	
Lys-Lys-Gly-Gln	

Fig. 3. The amino acid sequences of the enkephalins, and segments of  $\beta$ -lipotropin with morphine-like activity, numbered according to amino acid sequence of  $\beta$ -lipotropin.

catabolism, and the sites of their biosynthesis and action, await further study. It is possible that the small peptides may be synthesized in many parts of brain by the usual ribosomal type of protein biosynthesis, or that they are synthesized without ribosomal participation (like glutathione), or that they are formed by hydrolysis of larger peptides synthesized in the anterior pituitary gland. In our laboratory, we have been able to radiolabel enkephalins *in vivo* by injecting  $^3\text{H}$ -amino acid precursors into the rat CSF and isolating labelled enkephalins from the rat brain after thirty minutes (100). These experiments do not shed light on the site of enkephalin formation, but do suggest that endorphins are readily formed in brain. An understanding of the role that endorphins play in the actions of opiates is in its infancy. This knowledge will be most useful because the synthesis, metabolism and function of the endorphin system must be involved in the mechanisms of action of narcotic analgesic drugs in the central nervous system.

## SUMMARY

1. Narcotic analgesic drugs act throughout the central nervous system and at some peripheral neuromuscular junctions.
2. Opiate receptors at these sites may be identified by characteristic stereospecific binding of narcotic agonists and antagonists.
3. The initial drug:receptor interaction occurs in synaptic membranes, and may be followed by transduction through the cyclic nucleotide system.
4. Dopamine, norepinephrine, serotonin, acetylcholine and other neurotransmitters play a role in the action of opiates, but none play a unique role.
5. The neuroregulatory systems in brain also are involved in the mechanisms of action of the opiates.
6. The naturally occurring endorphins can be expected to play an important part in the mechanisms of opiate action, tolerance and dependence.

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## 14. OPIATE RECEPTORS: ISOLATION AND MECHANISMS

Eric J. Simon

Department of Medicine, New York University Medical Center,  
New York, New York.

### INTRODUCTION

Research on the mode of action of narcotic analgesic drugs and the mechanism of the development of tolerance and physical dependence is one of the oldest of scientific pursuits. For many years the first step in the action of these drugs was postulated to be their binding to highly specific "receptor" sites. This specific drug-receptor interaction was thought to trigger certain chemical or physical changes leading to the observed pharmacological responses. The reason for such a receptor postulate was the striking structural and steric specificity of many of the actions of narcotic analgesics. Rather minor structural changes have major effects on the pharmacology of the molecule, leading in some instances to the formation of antagonists, drugs whose main action consists of counteracting the effects of other opiates. While the receptor postulate dates back two to three decades, the discovery of the existence of specific opiate receptors occurred only about three years ago.

### *DISCOVERY OF STEREOSPECIFIC BINDING SITES IN ANIMAL BRAIN*

Binding of opiates to tissue homogenates was demonstrated some years ago in our laboratory using equilibrium dialysis (1). However, attempts to measure specific binding, defined as binding of labelled dihydromorphine, sensitive to displacement by the specific antagonist, nalorphine, were unsuccessful.

More recently Goldstein et al. (2) were the first to utilize the property of stereospecificity to search for opiate receptors. A series of modifications of the Goldstein procedure led to the discovery of stereospecific binding sites in homogenates of rat brain independently and simultaneously in three laboratories (3-5). In our laboratory we used <sup>3</sup>H-etorphine, a narcotic analgesic of enormous potency (about 10,000 times as potent as morphine in rats), as our labelled ligand. The hope, which was realized, was that the great



potency of this drug might reflect, at least in part, high affinity for the receptor.  $^3\text{H}$ -etorphine of high specific activity was incubated with rat brain homogenate at very low concentrations ( $10^{-10}$ - $10^{-8}\text{M}$ ) in the presence of a large excess of either unlabelled levorphanol (L) or its inactive enantiomer, dextrorphan (D). The homogenate was centrifuged and the pellet washed twice with cold buffer by recentrifugation. Radioactivity in the washed pellet was determined by liquid scintillation counting. Stereospecific binding was defined as that portion of the binding that is prevented by excess L but not by D. Using this procedure, most of the binding (70-80%) was found to be stereospecific. More recently a more rapid filtration technique was used by Pert and Snyder (5) and this has been adopted in our laboratory. Typical recent results from our laboratory are shown in Table 1.

TABLE 1

*Stereospecific Binding Assays By Filtration  
Procedure - Typical Results*

Drug	Concentration (M)	D (CPM)	L (CPM)	D-L (CPM)	pmoles bound mg protein
Etorphine	$2.5 \times 10^{-10}$	4100	540	3560	0.09
	$1.0 \times 10^{-9}$	7800	2000	5800	0.15
Naltrexone	$1.0 \times 10^{-9}$	3013	560	2453	0.08
	$2.5 \times 10^{-9}$	5360	1340	4020	0.17

*PROPERTIES OF STEREOSPECIFIC OPIATE BINDING SITES*

The binding sites have to date been found only in the central nervous systems of vertebrates and in the innervation of certain other tissues known to be sensitive to opiates, such as the guinea pig ileum and the mouse vas deferens. The sites are tightly bound to cell membranes. Enrichment of stereospecific binding in the synaptosome fraction (4,6,7) suggests that the binding sites are primarily present near synapses. Whether they are located post or pre-synaptically has not yet been determined.

Binding of opiates is saturable and half saturation (a measure of affinity) occurs at concentrations that are comparable to the brain concentrations at which these drugs are thought to exert their pharmacological effects. In a number of studies (3,8,9) excellent correlation has been found between

the *in vivo* potency of a large number of opiates and their affinity for stereospecific binding sites.

The opiate binding sites are very sensitive to proteolytic enzymes, such as trypsin, chymotrypsin and pronase (3,10) as well as to a variety of reagents known to react with functional groups of proteins, the most thoroughly studied of which are reagents that react with sulfhydryl (SH) groups (11). A protein (or proteins) is therefore essential for stereospecific binding of opiates. Sensitivity to phospholipase A (10) suggests a role for phospholipids. All properties of the stereospecific binding sites so far examined are consistent with their being the recognition and binding components of pharmacologically important opiate receptors. These sites will henceforth be referred to as receptors.

#### *DISTRIBUTION OF OPIATE RECEPTORS IN THE BRAIN*

The availability of human brain tissue obtained during autopsies at the Office of the Chief Medical Examiner of the City of New York, permitted us to establish the existence of opiate receptors in human brain. A study of the distribution of receptor sites in over forty anatomical regions of human brain was undertaken in collaboration with Dr. John Pearson (12). Levels of binding were found to vary greatly from region to region. High binding (0.3-0.4 pmoles/mg protein) was found in all regions of the limbic system except the hippocampus which has a rather low level of opiate binding. All regions consisting primarily of white matter, the cerebellum and regions of the brain stem were very low or virtually devoid of binding. High binding was also found in certain non-limbic areas such as the locus coeruleus and the pulvinar. Very similar results were reported by Kuhar et al. (13) for monkey brain.

#### *THE POSSIBLE PHYSIOLOGICAL ROLE OF OPIATE RECEPTORS*

Many investigators have suggested that, in order to survive the eons of evolution, opiate receptors must possess a physiological function that conveys a selective advantage on the organism that carries them. This led to a search for an endogenous ligand for the receptor. Exploration of all known neurotransmitters or modulators met with uniformly negative results. This then prompted the search for the existence of a previously unknown opiate-like molecule in the brain. Two laboratories were successful about the same time in demonstrating opiate-like activity in aqueous extracts of pig brain. Hughes et al. (14) showed that such extracts inhibited electrically stimulated contractions of isolated guinea pig ileum and mouse vas deferens. This inhibition was reversed by

naloxone. Terenius and Wahlstrom (15) showed that something in aqueous brain extracts was able to compete with labelled opiates for binding to opiate receptors. More recently Hughes et al. (16) reported that the active principle of their extract consists of two pentapeptides with the structures H-Tyr-Gly-Gly-Phe-Met-OH and H-Tyr-Gly-Gly-Phe-Leu-OH. A larger peptide with opiate-like properties was found in bovine pituitary by Goldstein and his collaborators (17,18). These opiate-like peptides, or endorphins, are discussed in detail by Dr. Goldstein. The question of their physiological role has not yet been answered. However, it is attractive to speculate that the endorphins and their receptors represent components of an endogenous pain-suppression system. At any rate, if a function is found for these peptides, the physiological role of opiate receptors will be clarified. For further detail see Loh and Law (chapter 19 in this volume).

#### CONFORMATIONAL FORMS OF THE OPIATE RECEPTOR

The findings in our laboratory (3) that increasing salt concentrations resulted in the reduction of opiate binding while no such effect was seen by Pert and Snyder (5) for naloxone binding, led us to suggest that this might reflect a difference in the manner in which agonists and antagonists bind to receptors. Evidence to support such a difference was obtained by Pert et al. (19) who also discovered that the effect was highly specific for sodium salts which reduced the binding of agonists but increased the binding of antagonists. Other alkali metal ions such as  $K^+$ ,  $Rb^+$  and  $Cs^+$  do not exhibit this discriminatory effect, while  $Li^+$  is partially effective. Detailed studies of the effect of sodium (20) led to the finding that the results are most readily explained by the interconversion of two conformational forms of the receptor. The conformation prevalent in a media containing  $Na^+$  has a higher affinity for antagonists and a lower affinity for agonists than the conformation that exists in  $Na^+$ -free media. Independent evidence for this interconversion was obtained by a study of the kinetics of receptor inactivation by the SH-reagent, N-ethylmaleimide (NEM) (11). As shown in Figure 1, in the presence of sodium, SH-groups of the receptors are markedly less accessible to inactivation ( $t_{1/2}$  of 30 min instead of 8 min). This protection shows the same ion specificity (Table 2) and response to sodium (Figure 2) as the changes in ligand affinity. The significance of the ability of the receptor to change its conformation is not yet known, but the great specificity of sodium in producing this phenomenon leads us to suspect that it may be important.

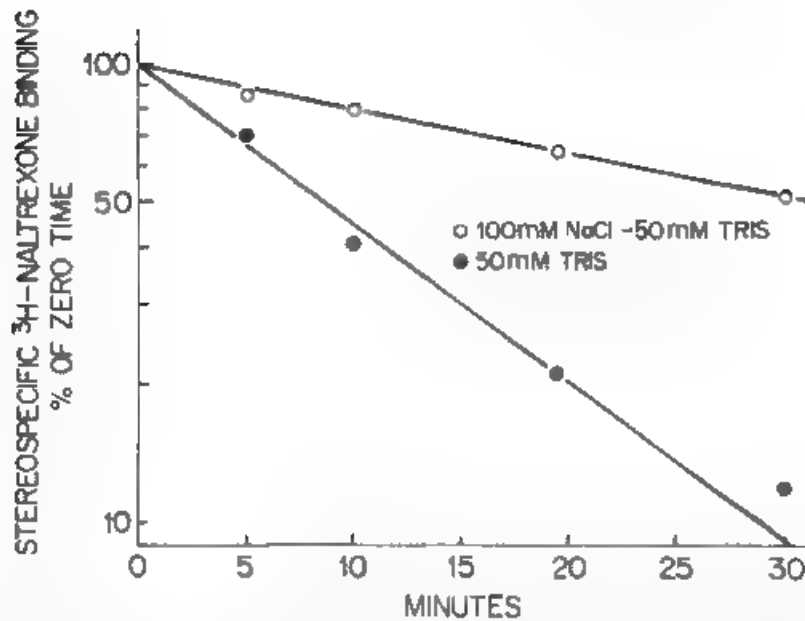


Fig. 1. Kinetics of inactivation of stereospecific  $^3\text{H}$ -naltrexone binding by NEM and protection by pretreatment with naltrexone. Inactivation was carried out at 37 degrees.  $P_2$  fraction was preincubated for five minutes with or without unlabelled naltrexone (3 nM) before addition of NEM. For the binding assay  $^3\text{H}$ -naltrexone was added to final concentration of 2 nM (total concentration of naltrexone 5 nM). From reference 11.

TABLE 2

*Effect of Alkali Metal Cations on Inactivation of Receptor Binding Capacity by NEM*

Time of preincubation (min)	Binding of $^3\text{H}$ -naltrexone % of zero time					
	Tris only	KCl	RbCl	CsCl	LiCl	NaCl
5	73	72	75	--	81	85
10	45	51	51	50	65	78
20	22	24	27	24	42	65
30	12	11	17	12	32	48

#### ATTEMPTS TO ISOLATE OPIATE RECEPTORS

To study the detailed chemical composition and functioning of opiate receptors it will be necessary to solubilize them off cell membranes and purify them. Some progress in this direction has been achieved in our laboratory (21). Cell membranes from rat brain are allowed to bind  $^3\text{H}$ -etorphine. The bound membranes are freed of unbound drug and concentrated

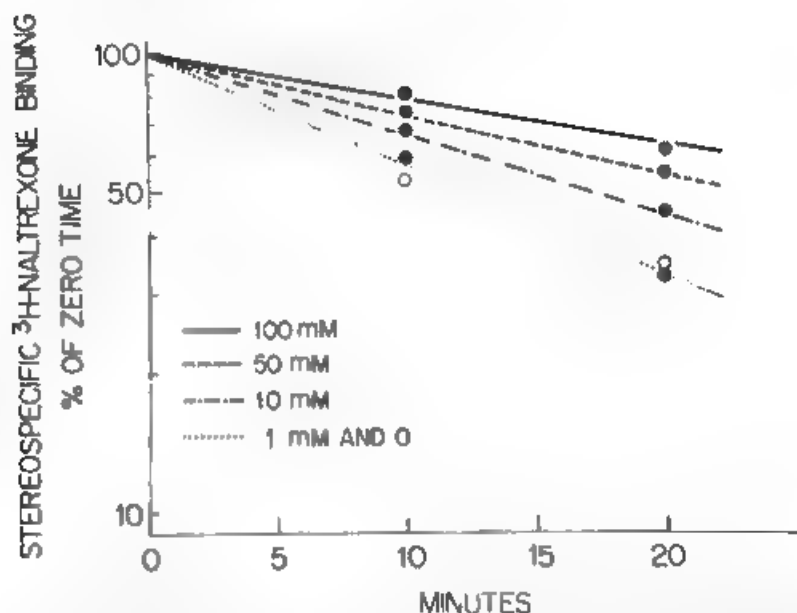


Fig. 2. Kinetics of inactivation of stereospecific binding of  $^3\text{H}$ -naltrexone by NEM in the presence of various concentrations of NaCl. Incubations with NEM were carried out in the concentration of NaCl shown. From reference 11.

by centrifugation and re-suspension in a smaller volume of buffer. The membranes are then treated with a 1% solution of the non-ionic detergent BRIJ 36T. Ultracentrifugation of this suspension at  $100,000 \times g$  yields a clear supernatant that contains most of the radioactivity. By use of chromatography on XAD-4 resin it was determined that 25-30% of the radioactivity in the supernatant is still bound to a large macromolecule (Figure 3). The molecular weight of this complex, as determined by chromatography on Sepharose 6B, was 350,000 (Figure 4). Evidence was obtained that the solubilized macromolecular moiety attached to etorphine has properties identical to those of the opiate receptor. To date, the free macromolecule obtained by allowing the etorphine to dissociate is not able to rebind opiates stereospecifically. Efforts to modify the conditions to allow us to obtain a soluble receptor capable of binding opiates in solutions are in progress.

#### SUMMARY

Considerable evidence has been accumulated demonstrating that stereospecific binding sites for opiates and their antagonists are the long-sought opiate receptors that mediate the pharmacological effects of these drugs. To date evidence suggests that one type of receptor can exist in several conformational states, but the question of the existence of

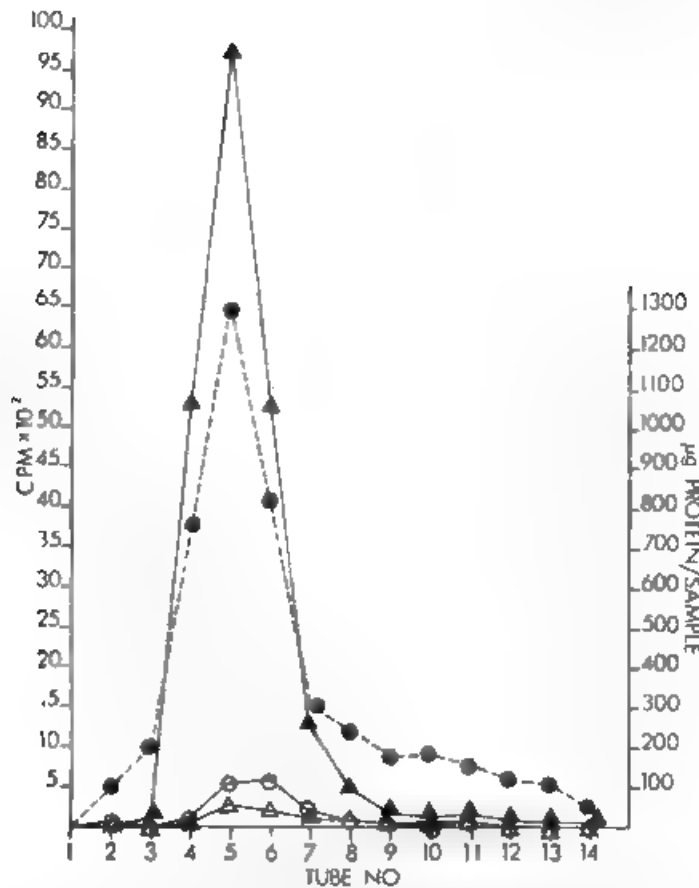


Fig. 3. Elution profile on XAD-4 of Brij extract of  $P_2$  membranes bound with  $^3\text{H}$ -etorphine.  $P_2$  membranes (2 mg of protein per milliliter) were incubated with  $^3\text{H}$ -etorphine ( $1 \times 10^{-9}\text{M}$ , 20.7 Ci/mmol) and subsequently extracted with 1% Brij 36T. A 1-ml sample of the supernatant, after ultracentrifugation, was placed on a column (2 by 10 cm) of XAD-4 (Rohm and Haas) and eluted with cold 0.05M tris buffer. (Δ)  $^3\text{H}$ -etorphine bound in the presence of  $10^{-6}\text{M}$  dextrorphan. (○)  $^3\text{H}$ -etorphine bound in the presence of  $10^{-6}\text{M}$  levorphanol. (◊)  $^3\text{H}$ -etorphine added to Brij extract of  $P_2$  membranes subsequent to extraction and ultracentrifugation. (○) Protein concentration is given in micrograms per milliliter. Data from reference 21, copyright 1975 by the American Association for the Advancement of Science.

multiple receptors for the many responses evoked by opiates is still unsettled.

The discovery of opiate receptors has recently given rise to another very exciting finding, namely, the existence of polypeptides in animal and human brain that can bind to the receptors and exhibit opiate-like activities. The study of the interaction of receptors with exogenous and endogenous ligands. The reactions triggered by these interactions, and

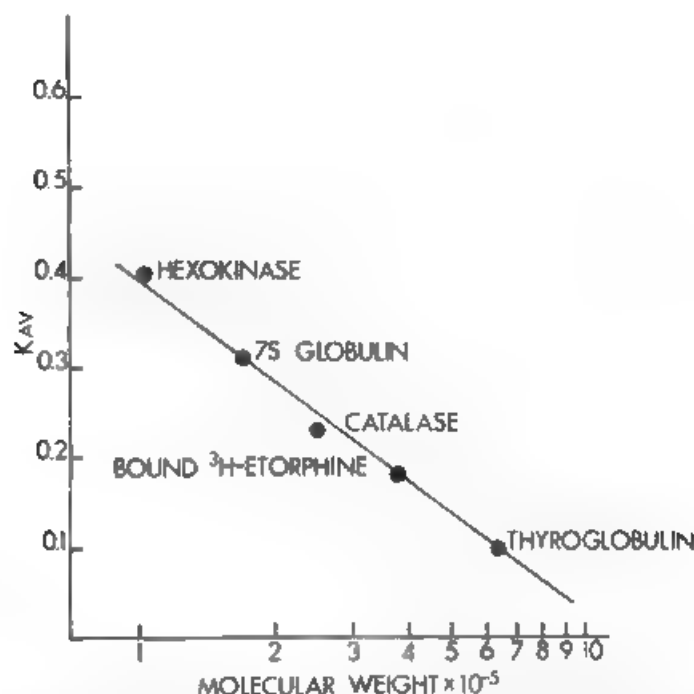


Fig. 4. Estimation of the molecular weight of solubilized  $^3\text{H}$ -etorphine-bound complex on Sepharose 6B. Gel filtration was carried out on a column, 1 by 52 cm; the eluting solution was 0.05M tris buffer, pH 7.4. Data are expressed as  $K_{av}$ ; by definition  $K_{av} = (V_e - V_o) / (V_t - V_o)$ , where  $V_e$  is the elution volume corresponding to the peak concentration of solute (marker proteins monitored by absorbance at 280 nm,  $^3\text{H}$ -etorphine-bound complex monitored by radioactivity determination),  $V_o$  is the void volume as determined by the appearance of dextran blue, and  $V_t$  is the total liquid volume as determined with free  $^3\text{H}$ -etorphine.  $V_o$  and  $V_t$  values were 20 and 65 ml, respectively. The relation between the logarithm of the molecular weight and  $K_{av}$  was used to obtain the molecular weight of the  $^3\text{H}$ -etorphine-bound complex. Data from reference 21, copyright 1975 by the American Association for the Advancement of Science.

the isolation and purification of receptor molecules should within the foreseeable future give us considerable insight into the mode of action of narcotic analgesics. An understanding of the physiological role of the receptor and its endogenous ligands may also lead to greater comprehension of aspects of brain function.

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## 15. IONS, OPIATES AND CELLULAR ADAPTATION

David H. Ross, Sherwood C. Lynn, Jr. and H. Lee Cardenas

Departments of Pharmacology and Psychiatry, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, 78284.

The pharmacological actions of opiate drugs are best characterized by sedation, analgesia, respiratory depression and changes in temperature. Repeated exposure to opiates, however, produces tolerance to these effects such that the magnitude of response is much reduced or absent. From this it must be assumed that the binding of opiates to their receptor sites activate certain biochemical events resulting in the observed pharmacological responses outlined above, as well as initiating steps leading to tolerance and dependence.

The nature of the biochemical steps producing tolerance is unknown. Earlier proposals (1,2) have been directed toward a study of metabolic adaptation by the cell, however, to date, pharmacokinetic observations of naive and tolerant animals have not accounted for the degree of adaptation to repeated drug exposure nor have they adequately explained physical dependence (3,4,5). A more functional approach attempts to explain tolerance and dependence based on neurochemical adaptations by the cell to repeated opiate exposure.

Theories offered by Shuster (6), Collier (7), and Goldstein and Goldstein (8) define cellular adaptation in terms of enzymes, membrane receptor expansion and feedback mechanisms associated with neurotransmitter synthesis. Collier's (7) hypothesis suggested that the mechanisms underlying the development of tolerance and dependence involved either a change in the number of receptors and/or their ability to bind opiate ligands. As tolerance develops, opiate agonist sensitivity (reduced) becomes inversely proportional to opiate antagonist sensitivity (increased). It is therefore of interest to view these sensitivities in terms of altered receptor activity, however, several laboratories (9-12) to date have failed to provide evidence for quantitative or qualitative changes in opiate receptors during chronic opiate treatment. Alternatively, one must therefore consider other mechanisms for induction of cellular adaptation leading to tolerance and dependence.

Recent advances in opiate receptor isolation and characterization have prompted a more molecular approach to study of cellular adaptation. The binding of opiate ligands to their respective receptor sites may stimulate membrane receptors which can communicate intracellularly to activate a number of major systems whose cumulative effects generate tolerance. This type of activation process may be similar in nature to the hormonal activation of membranal adenyl cyclase to increase the intracellular levels of cAMP. However, cellular adaptation to opiates may occur on an even more fundamental level requiring changes in a cellular constituent which serves to integrate broad areas of cellular activity.

#### *CALCIUM AS A MODULATOR OF CELLULAR FUNCTION*

Cell calcium functions in neurotransmitter activity and membrane stability and together with cyclic nucleotides, is thought to play a major role in regulating intracellular metabolism. By virtue of its obligatory involvement with cyclic nucleotides (13,14) and its role in neurotransmitter systems, Phillis (15,16) has suggested  $\text{Ca}^{++}$  may function as a primary and secondary messenger in the central nervous system. Table 1 outlines some of the areas of major involvement of  $\text{Ca}^{++}$ , many of which are also directly or indirectly affected by opiate treatment. Calcium activates tryptophan and tyrosine hydroxylase as well as adenyl and guanylyl cyclase and phosphodiesterase activities (17). Calcium is also an obligatory requirement for excitation-contraction (23) and secretion coupling mechanisms (24) and neurotransmitter-receptor interactions (25,26). Its role in membrane stabilization, activation of neurotransmitter release (by functioning as a charge carrier), and preliminary association in the regulation of macromolecule synthesis (27-31) further support a role for cell constituent in cellular adaptation.

#### *CALCIUM AND OPIATE ACTIONS*

##### Pharmacological Studies

Kakunago et al. (33) reported that intracisternal injections of  $\text{Ca}^{++}$  but not other ions  $\text{Ba}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Sr}^{++}$  or  $\text{Zn}^{++}$  nor  $\text{Na}^+$  or  $\text{K}^+$  antagonized opiate induced analgesia. Further EDTA or citrate antagonized  $\text{Ca}^{++}$ 's ability to alter analgesia. This finding was more recently confirmed by Harris et al. (34) who demonstrated that  $\text{Ca}^{++}$  antagonism of opiate analgesia is sensitive to EGTA but not EDTA. These investigators also demonstrated that lanthanum, a well-known  $\text{Ca}^{++}$  antagonist (35) may have a neuroanatomical site of action similar to morphine in producing analgesia and in fact can substitute for morphine

TABLE 1

*Multiple Role of Calcium in Biological Systems*

Biologic Function	System	Reference
Regulation of Enzyme Activity	Tyrosine hydroxylase	(18)
	Tryptophan hydroxylase	(17)
	Adenyl cyclase	(19,20)
	Guanyl cyclase	(21)
	Cyclic nucleotide Phosphodiesterase	(22)
-----		
Coupling Mechanisms	Excitation Contraction	(23)
	Excitation Secretion	(24)
-----		
Macromolecule Synthesis	Inhibition of aminoacyl t-RNA synthetase	
	Liver	(30)
	E. Coli	(29)
	Brain	(31)
-----		
Neurotransmitter Function	Neurotransmitter release	(28)
	Neurotransmitter receptor Interactions	(25,26)
-----		
Membranes	Membrane stabilization	(27)
-----		
Messenger Systems	Primary messenger CNS	(16)
	Secondary messenger PNS	(13)

in a cross-tolerance situation in producing analgesia (36,37). These later findings support earlier work by Shikimi et al. (38) and his suggestion that the analgesic action of morphine may be due to the opiate's affect on  $Ca^{++}$  flux. While  $Ca^{++}$

was the only cation found to alter analgesia,  $Mg^{++}$  content was observed to increase after acute opiate treatment (39).

Shikimi et al. (40) demonstrated morphine decreased whole brain  $Ca^{++}$  in mice, an effect to which tolerance developed but was independent of analgesia tolerance. In accordance with this finding was the report by Marchand and Denis (41) of increases in urinary excretion of  $Ca^{++}$ .

Studies in our laboratory have extended the work of Shikimi et al. (40) by demonstrating opiates in a dose dependent fashion cause  $Ca^{++}$  decreases in the brain in a fairly uniform manner (42). Many hypotheses regarding neurochemical basis for opiate tolerance and dependence regard primary mechanisms in terms of various neurotransmitters which may mediate the tolerance-dependence phenomena. Thus, it would be expected if a particular transmitter were involved, opiate effects may be seen predominately in that brain region where the neurotransmitter distribution is the greatest. Our data based upon assay of  $Ca^{++}$  loss in eight regional brain areas (43) would indicate no predominant area of opiate induced  $Ca^{++}$  loss suggesting a lack of correlation between  $Ca^{++}$  depletion and opiate effects on any transmitter system. Kuhar et al. (44) reported similar lack of correlation between opiate receptor binding and lessioned neurotransmitter regions. Further treatment with maximal  $Ca^{++}$  depleting doses of reserpine (5.0 mg/kg) and morphine (50 mg/kg) demonstrated additive  $Ca^{++}$  depletion indicating that morphine and reserpine sensitive  $Ca^{++}$  pools were in all likelihood distinct. In support of this finding Ross and Lynn (45) and Ross (46) showed reserpine pretreatment had no effect upon development of four hour and seven day tolerance to  $Ca^{++}$  depletion.

The depletion of  $Ca^{++}$  by morphine sulfate was also seen with the opiate congener levorphanol but not with the analgesically inactive (+) isomer dextrorphan. Naloxone effectively blocked the morphine induced decrease in  $Ca^{++}$  but did not prevent reserpine or pentobarbital from reducing calcium levels (47).

#### Relationship of $Ca^{++}$ to Development of Tolerance and Dependence

Many factors have been reported to influence the development of tolerance and a dependence on opiate drugs. Among the more prominent are those dealing with use of nucleic acid and protein synthesis inhibitors. Inhibitors of protein synthesis such as cycloheximide and puromycin have been shown to effectively alter development of analgesic tolerance (48). Tolerance to calcium depletion was observed by Shikimi et al. (40) and Ross (46) demonstrated that cycloheximide but not chloramphenicol effectively blocked tolerance to calcium

depletion. These studies are summarized in Figure 1. These results together with the lack of effects of reserpine pretreatment suggest two important points. Cycloheximide has been reported to have a locus of action directed to interruption of nerve membrane synthesis (49,50) while chloramphenicol's action is directed toward mitochondrial synthesis. Based upon these findings, rapid tolerance to  $\text{Ca}^{++}$  depletion may be explained by changes in synthesis of nerve membrane protein. Secondly, lack of reserpine's effect upon the induction of  $\text{Ca}^{++}$  tolerance suggests the locus of action for tolerance development may reside at membrane sites other than those sensitive to neurotransmitter stimulation.

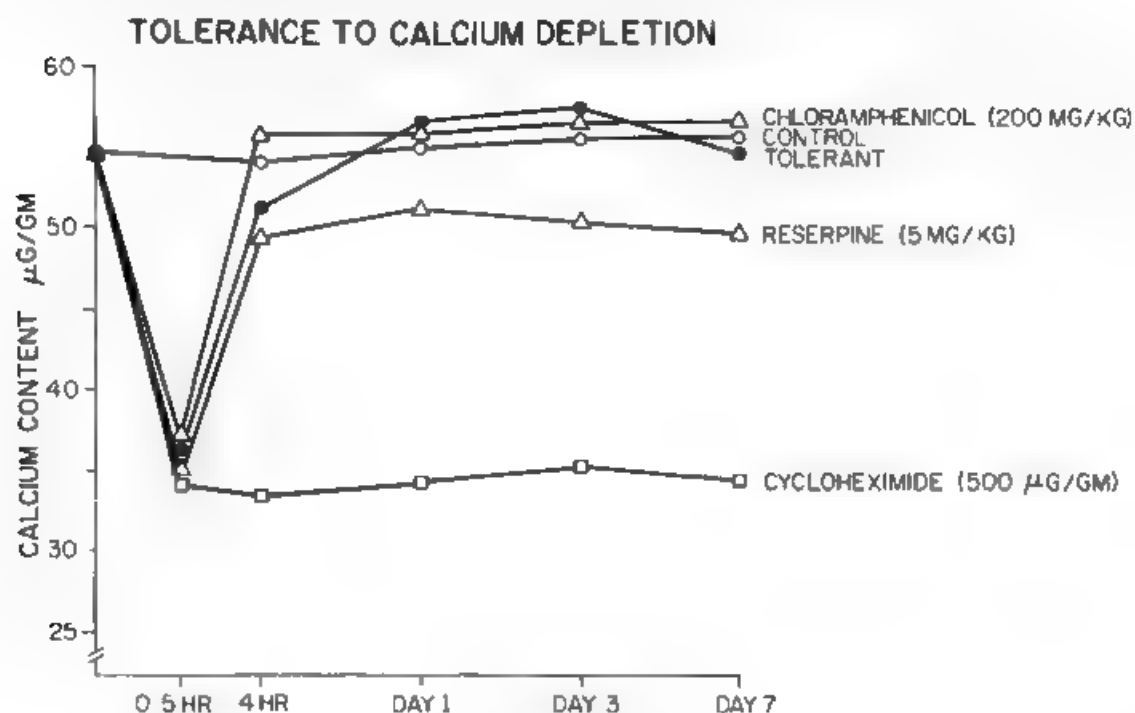


Figure 1

#### Subcellular Studies: Locus of $\text{Ca}^{++}$ Depletion

The earlier work of Shikimi et al. (40) and previous studies in our laboratory satisfy the criteria necessary to demonstrate calcium depletion as a specific opiate effect. However, it was of interest to us to further explore the locus of this calcium depletion with reference to specific subcellular calcium pools. If the calcium depletion is to be formally considered a specific effect of opiate treatment, one may expect that calcium levels would be altered in those subcellular fractions shown to preferentially bind opiate ligands. Pert and Snyder (51) initially reported that  $^3\text{H}$ -opiate ligands were predominantly bound *in vitro* to crude

mitochondrial fractions ( $P_2$ ) containing nerve endings, with less binding in microsomal ( $P_3$ ) fractions. Pert et al. (52) extended these observations by fractionating the crude mitochondrial fraction over sucrose gradients and demonstrating the majority of opiate binding to be associated with partially purified nerve ending fraction. More recent experiments, administering  $^3\text{H}$ -opiate ligands *in vivo* (53,54) have demonstrated association of the ligands with synaptic membrane fractions.

If the calcium decrease is the result of initial binding of the opiate agonist, causing displacement of  $\text{Ca}^{++}$ , the locus of the calcium decrease may be confirmed by examining  $\text{Ca}^{++}$  content in subcellular fractions after acute *in vivo* treatment. Cardenas and Ross (55) have examined the  $\text{Ca}^{++}$  contents in 11 subcellular fractions obtained by Ficoll-Sucrose gradients after acute opiate treatment. They report the locus of calcium depletion to be confined to the synaptic particulate fraction. A comparison of the subcellular distribution of opiate ligand binding (52) to calcium depletion may be seen in Table 2.

TABLE 2

*Comparison of Calcium Depletion and Opiate Receptor Binding in Subcellular Fractions of Rat Brain*

Subcellular Fraction	% $\text{Ca}^{++}$ Depletion (Cardenas & Ross, 1976)	% Opiate Bound (Pert et al., 1974)
$P_1$ (nuclear)	6.9	31
$P_2$ (crude mito)	90.9	52
$P_3$ (microsomal)	2.2	0
S (soluble)	0	17
-----		
Subfraction of $P_2^{++}$		
1) myelin	20	11
2) synaptosomal	73	67
3) mitochondria	4	22

\* fractions for opiate receptor binding prepared by sucrose gradient

+ fractions for  $\text{Ca}^{++}$  depletion prepared by Ficoll-Sucrose gradient

Crude separation of the subcellular fractions into  $P_1$  (nuclear),  $P_2$  (crude mitochondria),  $P_3$  (microsomal) and S (soluble) indicate a predominant loss of calcium from the  $P_2$  fraction. Similarly, but to a lesser extent, the predominant

binding of opiate ligands (52%) occurs in this fraction. Sub-fractionation of the crude mitochondria (containing myelin, synaptic nerve endings and mitochondria) by sucrose (52) or Ficoll-Sucrose (55) reveals the predominant calcium loss occurs in the synaptosome particulate (73%), with a small but significant loss occurring in the myelin (20%). This locus of calcium depletion is similar to the distribution of opiate receptor binding and supports the original premise that the calcium decrease occurs via displacement of synaptosomal  $\text{Ca}^{++}$  after opiate binding.

In addition to calcium, magnesium as well plays an important role in function of biological membranes and serves as cofactor for many enzyme systems (56,57). However, Kakunaga et al. (32) were unable to antagonize opiate analgesia with intracisternal injections of magnesium. More recently, it has been demonstrated both  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  could inhibit binding of opiate ligands to the crude membrane homogenate (10,51,58). Subsequently,  $\text{Na}^+$  and  $\text{Mn}^{++}$  were found to be the most effective *in vitro* modulators of opiate ligand binding (58-61). While  $\text{Ca}^{++}$  was found to be ineffective in differentiating agonist or antagonist binding.

Recent studies in our laboratory have examined the levels of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Mg}^{++}$  in subcellular fractions after a single dose of morphine. While calcium depletion was significant and confined to synaptic particulate fractions no changes in  $\text{Na}^+$ ,  $\text{K}^+$  or  $\text{Mg}^{++}$  were observed for any of the subcellular fractions (55). It is difficult to resolve at this time whether or not any endogenous cation may be regulating the conformation of a drug receptor. However, Lee et al. (62) have recently suggested, based on comparisons of opiate ligand binding in Tris-HCl vs. artificial buffers, that the presence of a combination of monovalent and divalent ions best contributes to affinity and accessibility of binding sites.

While the sodium model (58,59) is useful for *in vitro* discrimination of opiate ligand binding, the presence of 100 mM  $\text{Na}^+$  prevents physiologic uptake/release and binding of  $\text{Ca}^{++}$  to synaptic membranes (63-67). An alternative explanation for regulation of opiate ligand binding offered by Cardenas and Ross (55), views the opiate receptor in a  $\text{Ca}^{++}$  associated conformation. The binding of morphine induces  $\text{Ca}^{++}$  displacement shifting the membrane to a  $\text{Ca}^{++}$ -dissociated state which may be reversed by naloxone (47).

#### ADAPTATION TO SUBCELLULAR LOSS OF CALCIUM

As stated earlier, the binding of opiate ligands to their receptor sites is extremely sensitive to inorganic ions (51, 59). Calcium and magnesium, as well as sodium and lithium, but not potassium, have been reported to alter ligand binding



(10,58). If cellular adaptation at a membrane level is involved in the induction of tolerance to  $\text{Ca}^{++}$  depletion, it would appear that repeated administration of opiates may cause changes in one or more of the monovalent or divalent ions. Figure 2 illustrates the effects of tolerance and dependence upon calcium contents in synaptic particulate material. Twenty-four hours after morphine pellet implant  $\text{Ca}^{++}$  levels are significantly lower than control. Following seventy-two hours, calcium levels are significantly increased by 57% over control. The administration of naloxone to morphine pelleted mice reversed the elevated calcium to control levels.

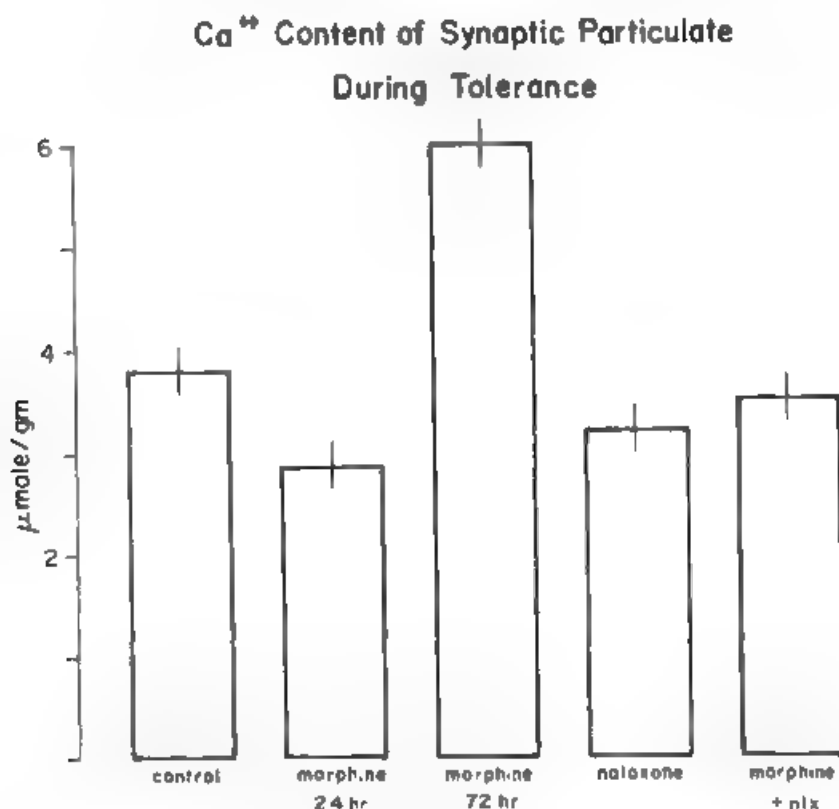


Figure 2

Table 3 outlines the results of similar studies which measured  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Mg}^{++}$  in synaptosomal particulate from morphine pelleted mice. These studies indicate that  $\text{Ca}^{++}$  contents are slightly lowered at twenty-four hours. No changes are reported for  $\text{Na}^+$ ,  $\text{K}^+$  or  $\text{Mg}^{++}$ . At seventy-two hours, magnesium was increased 13%. No changes were observed for  $\text{Na}^+$  or  $\text{K}^+$ . Naloxone induced withdrawal response produced a return to control of  $\text{Mg}^{++}$  levels while having no effect on the monovalent ions.

The slight but significant changes in magnesium observed at seventy-two hours are best described as indirect when

compared to changes in particulate calcium, since no changes in  $Mg^{++}$  are seen after acute opiate treatment (thirty minutes) (55) nor twenty-four hours after pellet implant. Pharmacological intervention using  $Mg^{++}$  to inhibit opiate analgesia was also without effect (32,34). These results would suggest that calcium content of the synaptic particulate significantly increases during the chronic exposure to morphine through pellet implant.

TABLE 3

*Cation Contents in Synaptic Particulate Fractions  
During Chronic Morphine Treatment*

	Cation Content $\mu$ moles/gm wet weight		
	Mean $\pm$ SEM		
	$Na^+$	$K^+$	$Mg^{++}$
Control	1.63 $\pm$ 0.03	1.25 $\pm$ 0.15	0.51 $\pm$ 0.04
Morphine (24 hr)	1.52* $\pm$ 0.08	1.06 $\pm$ 0.03	0.49 $\pm$ 0.08
Morphine (72 hr)	1.66* $\pm$ 0.08	1.56 $\pm$ 0.08	0.67** $\pm$ 0.03
Naloxone (72 hr)	1.73 $\pm$ 0.14	1.75 $\pm$ 0.57	0.56 $\pm$ 0.07
Morphine + Naloxone (72 hr)	1.83 $\pm$ 0.20	1.50 $\pm$ 0.30	0.51 $\pm$ 0.06

\* significantly different  $P > .01$

\*\* significantly different  $P > .05$

Means represent separate determinations from three separate experiments using three pooled mice brains in each experiment. Synaptosomal particulate was prepared according to methods outlined by Cardenas and Ross (1976). Cation contents were determined using the wet ash technique of Hanig et al. (1972). Pellets containing morphine base were implanted as described by Way et al. (1969). Groups of mice were sacrificed at twenty-four or seventy-two hours  $\pm$  ten minutes after 2 mg/kg naloxone. Cation contents were unaffected by the number of jumps the mice made at five, ten or twenty minutes (unpublished observation, Jones, D.J. and Ross, D.H., 1975). Control animals received lactose pellets  $\pm$  2 mg/kg naloxone at seventy-two hours.

A further insight into this increase in synaptic calcium content may be had by comparing the endogenous  $Ca^{++}$  content with  $Ca^{45}$  binding capacity of the membranes during various treatments with opiate drugs. In this way, the possible formation of new calcium binding sites may be detected by

observing the relationship between  $\text{Ca}^{40}/\text{Ca}^{45}$ . Table 4 illustrates the  $\text{Ca}^{40}/\text{Ca}^{45}$  associations with the synaptic particulate fractions. Naloxone effectively reverses the increase in  $\text{Ca}^{40}$  content after pellet implant to control. Acute opiate treatment produces decreases in  $\text{Ca}^{40}$  content, but an increase in  $\text{Ca}^{45}$  binding capacity.

TABLE 4

*Relation of Calcium Content to  $\text{Ca}^{45}$  Binding in Synaptic Particulate Fractions\* During Chronic Morphine Treatment*

	Calcium Content $\mu\text{moles/gm}$	Calcium Binding $\text{nmoles/mg protein}$
Placebo control	0.36	5.19
Morphine (acute, 30 min)	0.28	73.8
Morphine pellet (72 hours)	0.66	31.6
Morphine (72 hours) + NLX (4 mg)	0.31	56.3
Placebo + Naloxone (4 mg)	0.33	56.4

\* SPM fractions were obtained by methodology outlined in reference 55. NLX (4 mg/kg) administered at seventy-two hours and animals sacrificed ten minutes later. Preliminary experiments indicated jumping activity at five, ten or twenty minutes was independent of  $\text{Ca}^{++}$  content or binding.

These findings would suggest that acute exposure to opiates produces a membrane depleted state allowing more  $\text{Ca}^{45}$  to bind. Chronic exposure to opiates causes adaptation by the membrane in such a manner as to provide for new calcium binding sites. The increased  $\text{Ca}^{40}$  content during chronic treatment would thereby reduce the membrane capacity to bind  $\text{Ca}^{45}$ .

#### FUNCTIONAL IMPLICATION OF ALTERED $\text{Ca}^{++}$ METABOLISM

The increased  $\text{Ca}^{++}$  content and decreased  $\text{Ca}^{45}$  binding capacity of synaptic particulate fractions during chronic morphine exposure would suggest some adaptive response is occurring in the membrane. Collier (7) has presented a theory for the genesis of tolerance and physical dependence which postulates an increase in membrane receptors for opiates during tolerance. However, recent research has failed to

provide any direct evidence for an increase in narcotic receptors (9-12) during tolerance development. An alternative to Collier's hypothesis would consider activation of a membrane receptor which was biochemically linked to intracellular processes. Modification of membrane response could then activate an intracellular messenger system, leading to adaptation by the cell.

From a functional aspect, increased synaptosomal  $\text{Ca}^{++}$  content may be correlated with recent reports of increased neurotransmitter turnover and possible enzyme induction during development of opiate tolerance. Acute morphine treatment has been reported to increase dopamine turnover in striatal areas (68,69). This effect was found to be dose dependent and stereospecific (70,71) and tolerance was shown to develop (69) which paralleled pharmacological tolerance. Induction of tyrosine hydroxylase activity has also been reported (72) and with selective changes occurring in the rat striatum and hypothalamus. Since this enzyme is known to be rate limiting for the biosynthesis of dopamine and norepinephrine, it is of interest to note the recent reports of activation of tyrosine hydroxylase activity by physiological  $\text{Ca}^{++}$  concentrations. Morgenroth et al. (18) have demonstrated that physiological concentrations of  $\text{Ca}^{++}$  in the range of 50  $\mu\text{M}$  significantly stimulate this enzyme in central noradrenergic neurons, while EGTA was found to activate the enzyme from dopaminergic neurons (73).

This apparent modulation of enzyme activity by the presence or absence of  $\text{Ca}^{++}$  may have special relevance considering the known capacity of norepinephrine, dopamine and  $\text{Ca}^{++}$  to activate adenylyl cyclase. Conceivably, the action of opiates may indirectly, by increased transmitter turnover, stimulate adenylyl cyclase. In addition, alteration in membrane  $\text{Ca}^{++}$  by opiates may directly affect the activity of adenylyl cyclase. Recent evidence suggests that cyclic nucleotides may play a predominant role in mode of action of narcotics (74). Opiates may also act directly upon the cyclase system as suggested by the work of Klee et al. (75) and Sharma et al. (76). The binding of opiates may, through alteration of membrane  $\text{Ca}^{++}$  activate a messenger system which may in turn activate, at the intracellular level, mechanisms for transport of cations, neurotransmitters and enzyme activities, or more importantly, directly regulate various cellular functions such as phosphorylation of nuclear and synaptic membrane protein.

#### SUMMARY

The functional relationship of  $\text{Ca}^{++}$  binding to cellular response is of great importance in understanding how the cell responds to its surrounding environment. Areas for future

research regarding interaction of narcotics with calcium metabolism must include studies on  $\text{Ca}^{++}$  binding protein regulation of cyclic nucleotides. The low concentrations of  $\text{Ca}^{++}$  required for activation of adenylyl cyclase and cyclic nucleotide phosphodiesterase (19,22) provide a basis for the suggestion that their activities *in vivo* are regulated by variations in intracellular  $\text{Ca}^{++}$  content.

Cellular adaptation to opiates appears to include selective changes in membrane  $\text{Ca}^{++}$  receptors, to accommodate the cell to continued exposure to opiates. A wide variety of cellular responses to opiates, such as enzyme regulation and phosphorylation of specific proteins may therefore be regulated through  $\text{Ca}^{++}$  binding protein control of second messenger systems.

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## 16. ROLE OF PHOSPHATIDYL SERINE IN THE OPIATE RECEPTOR\*

L. G. Abood, F. Takeda, and N. Salem, Jr.

Center for Brain Research and Department of Biochemistry,  
University of Rochester Medical Center, Rochester, New York,  
14642.

### INTRODUCTION

The molecular nature and configuration of the opiate receptor is a problem under intensive investigation in a number of laboratories (1,2,3). Our interest in the problem developed as a result of a finding of others (4) that a proteolipid fraction from brain may be responsible for stereospecific opiate binding observed in various preparations of brain tissue. Upon examining various lipids, proteolipids, and proteins from membranes derived from brain, it was found that phosphatidyl serine (PS),† the major acidic lipid in brain, exhibited stereospecific opiate binding (5). Since the  $K_d$ 's for the opiate-PS complex and the opiate-tissue complex differed by three orders of magnitude, it was recognized that the binding to PS was nonspecific and different from the high affinity binding to membrane preparations. What interested us was the possibility that PS, in the form of a complex with a membranous protein, may be an important component of the opiate receptor.

In an effort to test this hypothesis a study was undertaken on the effect of added phospholipids on opiate binding. It had been known for many years that exogenous lipids were capable of exchanging with endogenous ones with no apparent alteration in their natural functional or structural

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† The following abbreviations are used: PS = phosphatidyl serine, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PA = phosphatidic acid, PI = phosphoinositides, CTAB = cetyltrimethylammonium bromide, CPC = cetylpyridinium chloride, FSC = cationic "Zonyl" fluoro-surfactant (DuPont), SDS = sodium dodecylsulfate, DNDFB = dinitrodifluorobenzene,  $^3\text{H}$ -DHM =  $^3\text{H}$ -dihydromorphine.

characteristics (6). It was demonstrated that the addition of PS to suspensions of synaptic membranes significantly enhanced both high and lower affinity binding, the  $K_d$ 's without lipid being  $1.0 \times 10^{-9}$  M and  $5.7 \times 10^{-9}$  M and those with lipid being  $5.0 \times 10^{-10}$  M and  $3.8 \times 10^{-7}$  M respectively (7). Another observation of interest was that exogenous PE was inhibitory to opiate binding. The present report describes studies aimed at extending the initial findings on the PS-enhancement and to attempt to understand the role of PS and other phospholipids in opiate binding.

#### MATERIALS AND METHODS

The procedures for the preparation of synaptic membranes from rat brain are described elsewhere (7). The technique for measuring the binding of  $^3\text{H}$ -DHM was essentially that of Pert and Snyder (3) with slight modifications (7). Other details concerning materials and methods are described in the legends of details and in the text.

#### RESULTS AND DISCUSSION

##### ENHANCEMENT OF OPIATE BINDING BY VARIOUS PHOSPHOLIPIDS

A number of phospholipids associated with most biological membranes were tested for their enhancement effect of the binding of  $^3\text{H}$ -DHM to a membrane preparation of rat brain. The three acidic lipids, PS, PA, and PI, produced a 35%, 29%, and 28% increase respectively when added at a concentration of 100  $\mu\text{g}/\text{ml}$  (Table 1). PC was without any effect, while PE resulted in a 10% decrease in binding. In a previous study it had been reported that a commercial preparation (from egg yolk and 70% pure) of PA was inactive (7). However, the present sample of synthetic dipalmitoyl PA (Sigma Chemical Co.), which was over 98% pure, proved to be active. As described elsewhere (7) other lipids such as cholesterol, diglycerides, and cerebrosides were without effect, while some enhancement (10%) was observed with cerebroside sulfates.

##### ENHANCEMENT BY VARIOUS SPECIES OF PS WITH VARYING FATTY ACID COMPOSITION

Recently, Salem et al. (8) have succeeded in separating and purifying the various species of PS from rat brain. At least four distinct species were identified, and their fatty acid composition is described elsewhere (8). In an effort to determine whether the fatty acid profile of PS influenced the opiate binding of neural membranes, the various species were tested for their ability to enhance the binding of  $^3\text{H}$ -DHM to

TABLE 1

*Enhancement of Stereospecific Opiate Binding by Various Lipids*

Lipids	<sup>3</sup> H-DHM binding (cpm)	% change
none	990	--
PS	1340	35
PS-ester	1250	26
PA	1280	29
PI	1270	28
PC	1010	2
PE	890	-10

*Preparation of synaptic membrane preparation and experimental conditions for measurement of stereospecific opiate binding described elsewhere (7). Lipids (100 µg/2 mg membrane protein) were homogenized with membranes and incubated 15 minutes prior to addition of <sup>3</sup>H-DHM (0.05 µCi/1.2 ml) incubation medium containing 50 mM Tris, pH 7.5 and 10<sup>-7</sup> M of either levorphanol or dextrorphan. Binding measured after filtration through GF/B glass fiber filters. Results expressed in counts/minutes (cpm), representing difference in radioactivity in presence of dextrorphan and levorphanol.*

membrane preparations (Table 2). The 18:1, and 18:0-PS were as effective as the PS mixture in enhancing opiate binding, while the 18:0, 22:4 and 18:0, 22:6-PS were less active. As will be described in a subsequent publication the degree of unsaturation in the aliphatic group is an important factor in the lipid enhancement of opiate binding. Although the 22:6 species is the major PS of synaptic membranes, some 18:1-PS is also present. The implications of these findings are presently under investigation.

**EFFECT OF VARIOUS PHOSPHOLIPASES ON OPIATE BINDING**

One approach to determining the role of lipids in opiate

TABLE 2

*Enhancing Effect of Various Species of Brain Phosphatidyl Serine on Opiate Binding*

Lipid	% Enhancement
none	--
PS (mix.)	35
PS (18:1)	34
PS (22:4)	10
PS (22:6)	15

*Lipids were prepared from bovine brain by argentation thin layer chromatography as described elsewhere (8).*

binding has involved the use of lipases. It has been reported that  $^3\text{H}$ -naloxone binding to rat brain homogenates is inhibited completely by a phospholipase A from *V. russelli*, 40% by phospholipase C (*Clostridium welchii*), and unaffected by phospholipase C (cabbage) (9). Although these observations suggest that phospholipids are involved in opiate binding, their interpretation is somewhat difficult. For example, the lysophospholipids formed by phospholipase A are strong detergents, which are themselves inhibitory. Another problem is the purity of the enzymes, which may contain proteases as well as various toxins.

We have been able to essentially confirm the findings of Pasternak and Snyder (9) utilizing a similar array of enzymes. After treatment of membranes with phospholipase C (*B. cereus*) opiate binding was reduced 45%, and although PS and PA were able to enhance binding after enzymic treatment, the activity was still below the control (Table 3). A combination of phospholipase D and C resulted in an 84% inhibition of opiate binding, while the addition of either PS or PA produced only a slight enhancement (Table 4). The effect of the lipases, therefore, appears to be irreversible, possibly because of a configurational change in the receptor caused by the lipase or other enzyme.

In an effort to determine the extent of enzymic degradation of the membranes by phospholipase C, an analysis was made of the membrane lipids. Even after exposure of the

TABLE 3

*Effect of Various Lipids on Opiate Binding After Treatment with Phospholipase C*

Lipid added	<sup>3</sup> H-DHM binding	% change
none	252	--
PS	385	35
PA	360	30
PS-ester	257	2
PC	255	2
PE	240	-5

Membranes were incubated with 0.5 unit of phospholipase C (*Bacillus cereus*) per mg membrane protein for one hour at 35° in 50 mM CaCl<sub>2</sub> - 50 mM Tris buffer, pH 7.5. Membranes were centrifuged at 100,000 x g, washed once with 50 mM Tris - 1 mM EDTA, pH 7.5. Aliquots of membrane preparation were homogenized with 100 µg of each lipid and pre-incubated 15 minutes before addition of <sup>3</sup>H-DHM and 10<sup>-7</sup> M of either levorphanol or dextrorphan. The lipase caused a 45% inhibition in absence of added PS.

membranes for two hours at a concentration of 2 units/mg membrane protein, there was no detectable change in the lipid content, utilizing quantitative thin layer chromatography (5). Another possibility is that impurities present in the enzyme were responsible for the inhibition, for example, a nonenzymic inhibitor is present in a phospholipase C prepared from *Cl. welchii* (Sigma Chemical Co.). In any case, the findings to date with the lipases are difficult to interpret.

#### EFFECT OF DETERGENTS ON OPIATE BINDING

In attempting to solubilize the opiate receptor, a variety of detergents were employed and found to be inhibitory to varying degrees. The membrane preparation was assayed for stereospecific opiate binding after being exposed to a 0.01% solution of the detergent. The nonionic detergent

TABLE 4

*Effect of Various Lipids on Opiate Binding After Treatment With Phospholipases A and C*

	cpm	% change
control	1150	--
PLase C	635	-45
PLases A + C	184	-84
PLases + PS	287	-75
PLases + PA	280	-76
PLases + PC	192	-83

*Experimental conditions same as in Table 1 except for the addition of phospholipase A (Vipera russelli) at concentration of 2 units/mg membrane protein.*

triton X-100 produced a 60% inhibition of opiate binding at a concentration of 0.01%, while the three cationic detergents, CTAB, CTC, and FSC, at the same concentration resulted in a 39%, 21%, and 15% inhibition respectively (Table 5). With the anionic detergent, SDS, a 25% inhibition occurred. Upon the addition of PS to the detergent-treated membranes, a marked increase (40%) in binding was observed with CTAB, while little or no effect was observed with the other detergents. The detergent-solubilized material was analyzed for opiate binding after removal of the excess detergent of Sephadex G-25 columns, and only with the CTAB-solubilized material was activity detectable (data not shown).

#### **RESTORATION OF OPIATE BINDING WITH VARIOUS LIPIDS AFTER CATIONIC DETERGENTS**

In an effort to understand the mechanism by which cationic detergents were inhibiting opiate binding, various lipids were tested for their ability to restore binding (Table 6). After membranes were exposed to 0.01% CTAB for 30 minutes, they were centrifuged at 100,000 x g, washed with 50 mM Tris, pH 7.5, and then re-centrifuged. (As discussed



TABLE 5

*Effect of Detergents on Opiate Binding and Reversal by Phosphatidyl Serine*

	% control No PS	% change Added PS
control	--	31
CTAB	-39	40
CPC	-21	9
FSC	-15	6
dodecyl sulfate	-28	0
triton X-100	-60	2

*Results are expressed as % differences in stereospecific binding of  $^3\text{H}$ -DHM.*

earlier, this preparation has only 60% of the binding activity of CTAB untreated.) Of the various lipids tested only the two acidic phospholipids, PA and PS, were effective in restoring opiate binding. The PS-ester, although enhancing opiate binding of untreated membranes, was without effect, as were PE and PC.

On the basis of these results there are two possible mechanisms for the effect of cationic detergents on opiate binding. The detergent may be interacting either with an anionic site on the protein component of the receptor or with the associated PS. Acidic lipids, by combining with CTAB, may, thereby, permit the receptor to assume its natural configuration. Another possibility is that the CTAB occupies an anionic site, either on the protein or lipid, and its removal by an acidic lipid frees the site for the opiate. Since the PS-ester is ineffective after CTAB-treatment but not before, it might seem more likely that the acidic lipids were acting by removal of the CTAB, rather than by activation of sites not occupied by CTAB. As will be discussed, however, the PS-ester may still retain an anionic charge.

TABLE 6

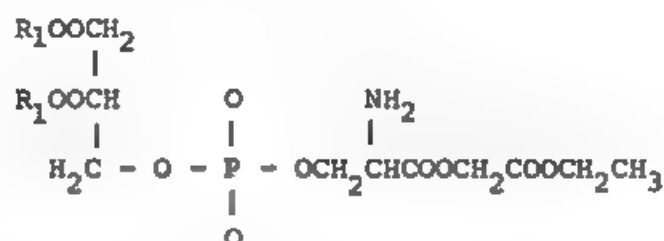
*Ability of Various Lipids to Restore Stereospecific Opiate Binding After Treatment with Cationic Detergent*

Lipid	d (cpm)	l (cpm)	d-l (cpm)	% change
--	571	329	242	--
PS	655	350	305	26
PA	751	381	370	53
PS-ester	519	315	204	-16
PE	560	330	230	- 4
PC	575	340	235	- 3

*Microsomes were treated with 0.01 CTAB and washed before exposure to lipids (0.1 mg/ml). Results are expressed as counts/minute, using  $10^{-7}$  M dextrophan (d) or levorphanol (l).*

#### ENHANCEMENT OF OPIATE BINDING BY AN ESTER OF PS

In order to determine whether the  $-\text{COO}^-$  group of PS was involved in the enhancement effect of the lipid, W. Hoss in this laboratory prepared an ester of PS from bovine brain PS, which has the following structure:



As is shown in Table 1, the PS-ester produced a significant enhancement effect on opiate binding. This finding suggests that the  $-\text{COO}^-$  group is not essential to opiate binding, and that the PS-ester is able to interact with the opiate receptor complex similarly to PS. Another possible explanation is that the PS-ester undergoes enzymic hydrolysis in the vicinity of the receptor and is converted to PS itself. It has

not been possible to demonstrate any hydrolysis of the PS-ester by the membrane preparation under the experimental conditions for opiate binding. As is discussed, however, the number of membranous PS molecules involved in receptor binding is extremely small. Therefore, the possibility of hydrolysis cannot be excluded. Another explanation for the effectiveness of the PS-ester is that the  $-PO^-$  is still available for interaction with either the opiate or protein, since the  $-C=O$  groups of the ester are capable of forming H bonds with the  $-NH_2$  group.

In the studies dealing with the ability of phospholipids to restore opiate binding after inhibition with CTAB, PS was effective while the PS-ester was not (Table 6). Such a difference supports the argument that the  $-COO^-$  group of PS is required for interaction of the bound CTAB, and although it favors the hypothesis that the PS-ester was not hydrolyzed, it is still conceivable that the extent of hydrolysis was insufficient to overcome the effect of CTAB. Nevertheless, the evidence to date favors the hypothesis that the PS-ester itself enhances opiate binding; therefore, the molecular configuration of the endogenous receptor complex has not been altered by the presence of the ester.

#### *EFFECT OF CROSS-LINKING OF AMINO GROUPS ON OPIATE BINDING*

By means of the reagent difluorodinitrobenzene (DFDNB), the amino groups of PS and, to a lesser extent, PE can be cross-linked to neighboring phospholipids and proteins within the erythrocyte membrane (10). In our laboratory it has been recently demonstrated that synaptic membranes undergo the same kinds of lipid-lipid and protein-lipid interactions observed for erythrocyte ghosts (10). After the exposure of neural membranes to 100  $\mu$ M DFDNB stereospecific opiate binding of  $^3H$ -DHM was decreased to 74% of the control (Table 7). Furthermore, upon the addition of PS to the DFDNB-treated membranes, there is no enhancement of opiate binding.

One interpretation of these observations is that the cross-linking of phospholipids to one another and to proteins resulted in either a partial structural alteration in the receptor complex or in restricting the availability of PS in the vicinity of the complex. The fact that exogenous PS did not enhance binding after cross-linking would indicate that either PS could not penetrate the membrane or gain access to the binding site, possibly because of the inability to exchange with endogenous phospholipids. It has been shown (10) that the erythrocyte membrane becomes refractory to hypotonic lysis after exposure to cross-linking reagents. Furthermore, the reagents tend to decrease the permeability of the membrane to anions (11).

TABLE 7

*Effect of Amino Cross-linking Reagent on Opiate Binding*

	<sup>3</sup> H-DHM binding (cpm)	% control	% increase by PS
control	920	--	--
control + PS	1223	133	33
DNDFB	684	74	--
DNDFB + PS	705	77	3

*Stereospecific <sup>3</sup>H-DHM binding was measured to synaptic membranes treated with 100  $\mu$ M DNDFB in 50 mM NaHCO<sub>3</sub>, pH 8.5 for one hour at 28° C, centrifuged at 100,000 x g, washed with 50 mM Tris, pH 7.5, and assayed for opiate binding.*

Preliminary experiments with the amino-crosslinking reagent, difluorodinitrobenzene indicate that PS is preferentially associated with protein in the synaptic membrane. When the covalently reacting reagent is incubated with a nerve ending preparation at pH 8.5 and room temperature, the lipid extract contains relatively large amounts of labelled PE and smaller amount of labelled PS. Similar results have been obtained in the red blood cell membrane (10). Quantitative analysis shows that 84% of the phospholipid crosslinked to protein is PS; the remaining 16% is PE. Further experiments are needed to establish a protein-PS complex in the synaptic membrane, however.

Other lines of evidence also suggest an interaction of PS with proteins. PS is known to activate enzymes such as Na<sup>+</sup>K<sup>+</sup> ATPase (12) and tyrosine hydroxylase (13). It is also interesting to note that brain PS is more effective in Na<sup>+</sup>K<sup>+</sup> ATPase activation than is PS prepared from egg yolk (12). Circular dichroism experiments have shown that PS can change the conformation of basic polypeptides (14) and nuclear magnetic resonance studies of intact bovine retinal rod outer segment membranes have suggested that a polyunsaturated species of PS is associated with protein, mainly rhodopsin (15).

### EFFECT OF $\text{Ca}^{2+}$ ON OPIATE BINDING

$\text{Ca}^{2+}$  has been shown to interfere with opiate binding (3) as well as the pharmacological effects of opiates (16). Furthermore, morphine inhibits the phospholipid-facilitated transport of  $\text{Ca}^{2+}$  (17) and results in a depletion of brain  $\text{Ca}^{2+}$  *in vivo* which is reversed by naloxone (18). From such observations it would appear that the pharmacological action of the opiates is related to that of  $\text{Ca}^{2+}$ . One possibility is that  $\text{Ca}^{2+}$  by combining with PS or other phospholipids, may either compete directly with the opiates for an anionic site on the receptor or modify the configuration of the receptor.

In order to examine this possibility, the enhancement of opiate binding by PS was compared with that occurring in the presence of the  $\text{Ca}^{2+}$  salt of PS. Only a slight enhancement (9%) was observed with  $\text{Ca}^{2+}$ -PS (Table 8). When 1 mM  $\text{Ca}^{2+}$  was present, the addition of PS failed to produce any enhancement of  $^3\text{H}$ -DHM binding. It is difficult to determine from these studies whether  $\text{Ca}^{2+}$  inhibits opiate binding by occupying an anionic site on the receptor independently of the lipid, or whether it interferes with the action of the phospholipid component by complexing with PS or other acidic lipids.

### POSSIBLE MECHANISMS FOR PS ENHANCEMENT OF OPIATE BINDING

Since PS by itself is known to interact stereospecifically with opiates, a question to consider is whether it can still do so as a normal constituent of the membrane. A comparison of the  $K_d$ 's for opiate binding of PS alone and membrane fragments reveals a difference of three orders of magnitude. Consequently, the binding characteristics of the two systems must be very different. There are, however, similarities between the two, including pH optimum and inhibition by divalent cations (e.g.,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) and by high ionic strength. It is likely that the molecular interactions existing in the membrane between PS and proteins are perturbed by the opiates and other exogenous ligands and that the molecular complementarity of the ligand involves a PS-protein complex. If it is assumed that PS is a component of the opiate binding site, then only a very small percentage of the total endogenous PS participates in binding.

If the amount of PS in whole rat brain is assumed to be about 10  $\mu\text{moles/g}$ , then the number of PS molecules would be about  $6 \times 10^{18}/\text{g}$ . From a Scatchard analysis the number of opiate binding sites/g of whole rat brain was estimated at  $10^{13}$  (19). Since the number of PS molecules exceeds the binding sites by 5 to 6 orders of magnitude, it must be concluded that the determining factor in binding is the number

TABLE 8

*Effect of  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$  Salt of PS on Stereospecific Opiate Binding*

	$^3\text{H}$ -DHM binding (cpm)	% change
control	820	--
+ 1 mM $\text{Ca}^{2+}$	430	-48
+ PS	1150	29
+ PS + 1 mM $\text{Ca}^{2+}$	830	1
with $\text{Ca}^{2+}$ -PS	900	9

*Experimental conditions were the same as described in Table 1 except that membranes were prepared in absence of EDTA.*

of specific proteins or other macromolecular species of which PS is a component.

In attempting to interpret the results of the various phospholipases on opiate binding, it is essential to first determine whether they are due to a removal of PS from the membrane or to a general effect on membrane structure produced by the chemical alteration of the phospholipids. The lysophospholipids resulting from exposure to phospholipase A are known to be strong detergents and their presence in the vicinity of the opiate receptor may be altering its configuration. If such were the case, then the experiment with phospholipase A could not be used to support the argument that phospholipids are part of the receptor complex. Although the results with phospholipase C are more supportive of the role of phospholipids in opiate binding, they do not exclude the possibility of indirect effects resulting from altered lipids.

In an effort to determine the extent of the phospholipid degradation resulting from exposure to phospholipase C and D, analyses were made of the lipid content of the membranes. With phospholipase D, which had no effect on opiate binding, no alteration in lipid content of the membranes was detectable even after prolonged exposure to relatively high concentrations of the enzyme. Although phospholipase C had markedly inhibited opiate binding, the alteration in the lipid com-

position was barely detectable, being less than 1-2%. When a combination of phospholipase A and C was used, the extent of hydrolysis was less than 5%. It appears that neural membranes are considerably more resistant to the combination of the two lipases than were erythrocyte membranes, where the hydrolysis was virtually complete (20). Despite the slight degree of lipid degradation by phospholipase C, the results may be particularly significant, since only a small fraction of the total membrane PS may be associated with the opiate binding site.

#### SUMMARY

A study was undertaken to investigate the possible role of phospholipids in opiate binding to brain membrane preparations, particularly with regard to phosphatidyl serine (PS). Upon the addition of lipids to suspensions of synaptic membranes it was found that PS and phosphatidic acid enhanced the stereospecific binding of  $^3\text{H}$ -dihydromorphine as much as 50%. Phosphoinositides were less effective, while other lipids were inactive. After partly inhibiting opiate binding by exposure of membranes to phospholipase C, it was still possible to enhance binding with PS, but not to restore it, whereas after treatment with phospholipase A + C, no enhancement occurred. Since the ethoxymethyl acetate ester of PS was as effective as PS in enhancing binding, it was concluded that the  $-\text{COO}^-$  group was not directly involved in opiate binding; however, the  $-\text{PO}^-$  group may be involved. Inhibition produced by the cationic detergent cetyltrimethylammonium bromide was completely reversible with PS and other acidic phospholipids, but not with the PS-ester. In the presence of 1 mM  $\text{Ca}^{2+}$  PS produced no enhancement. It is concluded that PS may be an essential component of the opiate receptor.

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## 17. OPIATES AND CYCLIC AMP

Werner A. Klee

Laboratory of General and Comparative Biochemistry, National Institute of Mental Health, Bethesda, Maryland, 20014.

Cyclic nucleotides, and particularly cyclic AMP, are believed to play important roles in the nervous system as second messengers of synaptic communication (1-4). Thus, catecholamines functioning through  $\beta$ -adrenergic receptors, dopamine, histamine, adenosine, E prostaglandins and some other putative neurotransmitters and neuromodulators are believed to function as substances which stimulate adenylate cyclase and thus raise cyclic AMP levels in cells with the appropriate receptors. Morphine and the other opiate-like substances have been shown to interact, directly or indirectly, with many of these transmitter and modulator substances (5-7), and it was therefore natural to ask whether the opiates might not also function via the adenylate cyclase system.

Studies by Collier and Roy (8,9) with brain homogenates provided the first biochemical evidence that morphine may function as a specific inhibitor of adenylate cyclase. These workers found that morphine, levorphanol, methadone, heroin, etorphine and pethidine, but not dextrorphan, inhibit the PGE<sub>1</sub> stimulated conversion of (<sup>3</sup>H)-ATP to (<sup>3</sup>H)-cyclic AMP in whole homogenates of rat brain, and that this inhibition is prevented by naloxone. These experiments demonstrated the appropriate specificity and pharmacological relevance of the inhibition of opiates of cyclic AMP formation in brain homogenates. Although a number of workers have not been able to demonstrate such inhibitory effects of opiates on cyclic AMP production in brain homogenates (10-12), recent work by Wilkenin and Makman (13) in homogenates of monkey amygdala confirms at least in large part, the results of Collier and Roy, as do some recent experiments with brain slices by Minneman and Iversen (14). Nevertheless, the situation in brain seems excessively complicated due, in part, to the large number of cell types and functional regions represented both in homogenate and in slice preparations. Regional heterogeneity of response in the brain is well demonstrated in experiments in which cyclic nucleotide levels are measured after *in vivo* manipulations (15,16).

In an effort to find a more homogeneous cell population with which to study the mechanism of morphine action, Klee and Nirenberg (17) screened a number of cell lines, which are maintained in continuous culture, for the presence of opiate receptors. One of the cell lines examined, neuroblastoma x glioma hybrid NG108-15, was found to possess a large number of morphine receptors ( $2-3 \times 10^5/\text{cell}$ ) and this cell was chosen for further study. The hybrid cells had been prepared (18) by virus-induced fusion of a mouse neuroblastoma cell line (clone N18-TG2) (19), and a rat glioma cell line (clone C6-BU1) (20), neither of which, interestingly, contains large numbers of morphine receptors. Clone N18-TG2 has subsequently been shown to contain a measurable, but still relatively small, number of receptors when a modified assay is used (21). The properties of the opiate receptor of NG108-15 hybrid cells are, in all ways tested (17) similar to those found in rat brain homogenates (22-26).

Sharma, Nirenberg and Klee (21) found that the adenylate cyclase activity of homogenates of NG108-15 is sensitive to inhibition by opioid agonists such as morphine, etorphine and levorphanol at concentrations which are consistent with the pharmacological potency of these drugs and which are also similar to the concentrations at which they bind to the opiate receptor (Table 1). Furthermore, dextrorphan, the inactive

TABLE 1

*The Relation Between Opiate Inhibition of Adenylate Cyclase and Receptor Affinity in Homogenates of NG108-15*

	Receptor Affinity <sup>a</sup> nM	K <sub>i</sub> Adenylate Cyclase nM
Etorphine	5	10
Levorphanol	200	200
Morphine	4,000	2,000
3-Allylprodine	10,000	50,000
Dextrorphan	10,000	(inactive)
Naloxone	20	(antagonist only)

a. Measured in reaction mixtures identical in composition with those used in the assay of adenylate cyclase, by displacement of (<sup>3</sup>H)-naloxone. Data from Sharma, Nirenberg and Klee (21).

stereoisomer of levorphanol does not inhibit the adenylate cyclase of these cells nor does the opiate antagonist, naloxone. The latter substance will, however, effectively antagonize the action of morphine and other opiates on adenylate cyclase, at concentrations which are consistent with the known affinity of naloxone for the opiate receptor. Furthermore, the adenylate cyclase activity of homogenates of the parental cell lines neuroblastoma N18-TG2 (containing few opiate receptors) and glioma C6-BU1 (containing no opiate receptors) is inhibited only slightly and not at all, respectively (21). Thus, these studies, which have been confirmed in other laboratories (10, 27), establish the functional linkage of morphine receptors as inhibitors to adenylate cyclase.

Study of cyclic AMP levels in intact NG108-15 cells by Traber, Hamprecht and their colleagues (28-31) as well as Sharma et al. (21,22) has led to the same conclusion. Cyclic AMP levels of these cells are raised many fold by prostaglandin  $E_1$  (32) or adenosine (33) and these increases are blocked by opiates in a naloxone reversible, and stereospecific way (29, 33). Basal cyclic AMP levels are also found to be lowered by opiates (21,33).

Endogenous opiate peptides, the enkephalins (34,35) and others (36-39), have been recently characterized and are the objects of extraordinarily intense investigations. These fascinating substances are undoubtedly of physiological importance (40,41) and apparently supply the answer to the question of the purpose for which opiate receptors exist in the vertebrate nervous system. It was natural, therefore, to ask whether the enkephalins and other endogenous opiate peptides function as receptor-mediated inhibitors of adenylate cyclase as do the standard opiates. These pentapeptides have been shown by Klee and Nirenberg (42) to be among the most potent receptor-mediated inhibitors of adenylate cyclase known and by Brandt et al. (43) to inhibit cAMP accumulation in NG108-15 hybrid cells. Their properties are much like those of morphine except that they are short acting, presumably due to rapid proteolytic degradation (44,45). Longer fragments of  $\beta$ -lipotropin as well as other peptides with opiate-like properties have also been shown to inhibit the adenylate cyclase of NG108-15 cells in a naloxone reversible manner (46), and sometimes with a somewhat longer duration of action than that of the enkephalin. We may expect studies in the future of the interaction of a series of related peptides with the opiate receptor and with adenylate cyclase to lead to new insights into the mechanism of action of these receptors. Certainly, the information which has emerged thus far needs to be supplemented with more detailed studies.

The problems of opiate tolerance and dependence are characteristic of all examples of this class of drug which

have been studied in sufficient depth. Studies of adenylate cyclase and cyclic AMP levels in neuroblastoma x glioma NG108-15 cells have also led to interesting new insights into these phenomena. Hybrid cells, when cultured for a number of hours in the continued presence of opiates (31,47) or of enkephalin (48,49) develop a compensatory mechanism which results in tolerance to the effects of these substances simultaneously rendering the cells dependent upon the presence of opiates for normal cellular function. The biochemical basis for this tolerance and dependence appears to be the development of an increased amount of adenylate cyclase activity (47). An experiment demonstrating the development of increased adenylate cyclase activity in response to cell culture in the presence of morphine is shown in Table 2, experiment 1. Enzyme activity is seen to increase to such an extent that, in the morphine treated cells, activity measured in the presence of added morphine is equal to that of control cells measured in the absence of inhibitor. In the assay, morphine, which had been present during culture of the cells has been removed by washing prior to homogenization. Cells which have responded to opiates or opiate peptides by a compensatory increase in adenylate cyclase activity are dependent upon opiates since the rapid withdrawal of these substances by means of the antagonist naloxone will result in dramatic increases in cyclic AMP levels to values well above those normally found (Table 2, experiment 2).

As we have seen, opiates have two types of effects upon adenylate cyclase in NG108-15 cells: an immediate inhibition followed by a slowly developing increase in total activity. This dual regulation (47) can account for all of the known effects of narcotics, although other contributory mechanisms cannot be ruled out at present. A diagrammatic summary of the dual regulation of adenylate cyclase by opiates is presented in figure 1. The figure shows that opiates produce an immediate fall in cellular cyclic AMP levels (panel A) due to inhibition of adenylate cyclase without change in its total activity (panel B). Upon continued exposure to opiates the cells respond by an increase in the total amount of adenylate cyclase activity which (even though the enzyme is still inhibited by morphine) brings cyclic AMP levels back to normal and the cells are now tolerant to opiates. An important feature of the opiate receptor mediated inhibition of adenylate cyclase, is that incubation of enzyme activity is never complete, even at saturating amounts of inhibitor (21). If inhibition of enzyme activity were complete, tolerance could not be produced by the mechanism described here except at low, subsaturating concentrations of drugs. The model also demonstrated that tolerant cells are dependent upon opiates since rapid withdrawal of morphine results in a release of the

TABLE 2

*Adenylate Cyclase Activity and Cyclic AMP Levels in NG-108-15 Cells Cultured in the Presence of Morphine (Data of Sharma et al., reference 47)*

Experiment 1                      Adenylate Cyclase Activity of Homogenates (pmoles CAMP/min/mg protein)		
Days in Culture	Additions to Assay	
With 10 $\mu$ M morphine	None	10 $\mu$ M morphine
0	11.8	6.0
2	19.8	9.6
3	25.2	12.6
4	31.2	14.2
-----		
Experiment 2                      Cyclic AMP Levels in Intact Cells (pmoles cAMP/mg protein)		
Days in Culture	Additions to Assay	
With 10 $\mu$ M morphine	None	10 $\mu$ M naloxone
0	20	21
2	23	37

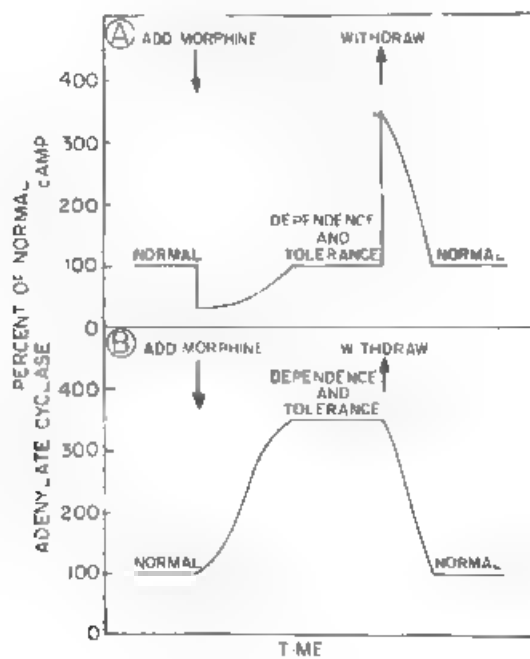


Figure 1

inhibition of an abnormally high amount of adenylate cyclase activity with the consequent formation of abnormally large amounts of cyclic AMP. The dual regulation model is consistent with the compensating hypertrophy proposed by Collier and Roy (8), and is a specific example of the enzyme inhibition and expansion models proposed by the Goldsteins (50) and Shuster (51) and by the homeostatic model proposed by Himmelsbach (52). It has many features which are testable in animals and some evidence that this type of mechanism operates in whole animals has been produced (16,53-56). On the other hand, many features of the model are, as yet, only poorly understood. Thus, neither the mechanism of the receptor mediated inhibition of adenylate cyclase activity nor that of the receptor mediated delayed positive response is understood.

Morphine receptors can now confidently be considered to be, in reality, polypeptide hormone (or neurotransmitter) receptors, and they are most probably located on the cell surface (17). Their coupling with adenylate cyclase may be via the same, as yet unknown, types of mechanisms which couple glucagon, ACTH or other peptide hormone receptors with the enzyme. Although there is some evidence which implicates protein synthesis in the mechanism of acquisition of opiate tolerance and dependence, it is not at all clear that the increased adenylate cyclase activity is due to the synthesis of an increased number of molecules of enzyme. Conceivably, the increase in activity observed may reflect altered control mechanisms and not more enzyme. These questions are currently being explored in a number of laboratories and their resolution should do much to increase our understanding of the mechanism of opiate action.

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## 18. PHARMACOLOGICAL HETEROGENEITY OF NARCOTIC RECEPTORS

Alfred A. Smith

New York Medical College, Department of Pharmacology, New York, New York, 10019.

### INTRODUCTION

The nature of opioid receptors has intrigued many investigators because a stereoisomeric requirement is coupled with an ability of structurally-related antagonists to inhibit virtually all the pharmacological actions of the opioids. Recent observations (1-3) using fragments of nerve endings or synaptosomes show low-saturability, high-affinity and stereospecific binding of radiolabelled narcotics or their antagonists. Demonstration of opiate binding material is considered evidence for the existence of an opiate receptor since none of a wide variety of non-opiate drugs, in low concentrations, were bound by receptor substances.

The highest binding capacity is associated with the limbic system of the brain. The same regional distribution of radiolabelled opioid was found in human as in monkey brains. Despite the remarkable relationship between binding affinities and opioid potency the binding properties of the receptor substance remain unchanged by induction of tolerance (4). However, Pert et al. (5) found that acute administration of opioids or antagonists slightly enhanced stereospecific binding capacity of brain extracts but this effect remained fixed despite morphine pellet implantation. They concluded that the enhanced receptor binding was unrelated to either tolerance or physical dependency and have suggested that neither tolerance or physical dependency bear any relationship to the narcotic receptor and that any change induced by chronic treatment is remote from the opioid receptor itself.

The fact that a morphine receptor exists prompted a search for an endogenous analog. Terenius and Wahlstrom (6) and Hughes (7), reported that low molecular weight peptides interact with the morphine receptor and this binding can be inhibited by narcotic antagonists. These findings have stimulated much new research.

Because some functions of the partially isolated narcotic receptor material appear disparate from pharmacological

function, it is essential to determine how opiate receptors behave in the intact animal and whether responses vary from one another. If such variations exist then it may be possible to distinguish among opiate receptors and the mechanisms by which these receptors may be modified. Quantitative pharmacological measurements can estimate the  $pA_x$  of the opioid receptor (8). This procedure expresses the activity of an antagonist in relation to agonistic action. When the subscript '2' is applied, the  $pA_2$  represents the negative log of the molar concentration of the antagonist needed to shift the dose response curve of the agonist two-fold to the right, implying that the antagonist now occupies half the receptor sites. Determination of the precise  $pA_2$  is always made *in vitro* where concentrations are known.

In order to obtain analogous data in the intact animal it is essential to obtain measurements during peak activity of the administered drugs. It is assumed there that the concentrations of the drugs are related to the dosage injected. Cox and Weinstock (9) utilized this procedure and reported on quantitative studies of the antagonism by nalorphine of various opioids. Their study showed that the apparent  $pA_2$  for transient cataract formation in the mouse eye was the same as the  $pA_2$  for analgesia. These investigators stated that on the basis of their findings, the receptor for cataractogenic effect and for analgesia must be the same.

Takemori et al. (10) showed that nalorphine or naloxone antagonized morphine analgesia and inhibition of the gastrointestinal tract, but through different receptors. The *in vivo* apparent  $pA_2$  for analgesia in mice was 7.01 and for intestinal inhibition, 6.6. That these receptors ought to be different is supported by the well-known observation that tolerance can develop to the analgesic effect of morphine but less easily to the constipating action of morphine. Other reasons for the observed difference include the probability that morphine has many actions on the gut including blockade of acetylcholine release and stimulation of 5-hydroxytryptamine. These investigators suggest that receptor types may be ranked using a series of agonists in several systems. Evidence for the existence of alpha and beta adrenergic receptors was developed by the same method. Miller and Anderson (11) did find differences in the relative potencies of the *nor*-derivatives of morphine, codeine and meperidine in the mouse, which suggested a difference in receptor types.

Takemori et al. (12) made the interesting and important observation that a single dose of morphine given only two hours before an antinociceptive test could significantly increase the apparent  $pA_2$ . The receptor was thus sensitized to the antagonistic action of naloxone an effect which persisted for as long as four days after the injection of the

morphine. Two methods for analgesic assay were undertaken. One involved the writhing test as modified by Hayashi and Takemori (13) and the other was the tail-flick procedure described by D'Amour and Smith (14). Both tests revealed that a previous injection of morphine produced a  $PA_2$  increase about three times greater than control within two hours after the injection of morphine. There was no increase in the  $ED_{50}$  for morphine itself indicating lack of tolerance development. Unlike morphine the mixed agonist-antagonist pentazocine did not induce a rise in  $PA_2$  suggesting that it had no effect on the conformation of the narcotic receptor. These investigators suggested that only narcotic analgesics cause a structural change in receptors with increased affinity of the receptors for antagonists. They concluded that their findings may help explain why narcotic antagonists are so effective against severe narcotic depression but not as effective in mild depression.

Our interest in the possible heterogeneity of narcotic receptors was prompted by our observation (15) that a single dose of levorphanol induced marked, long-term tolerance to the cataractogenic effects of the opioid whereas the respiratory response to levorphanol remained unchanged after a similar large dosage of the drug. Furthermore, we discovered that the administration of reserpine did not alter the response to the respiratory depressive effect of levorphanol, although a marked decrease was obtained in its antinociceptive action. These observations made it seem unlikely that the receptor mechanisms could be identical. In addition, cataractogenic tolerance occurred quite rapidly and persisted for long periods of time unlike analgesic or respiratory tolerances. We therefore undertook a systematic study of each of these three characteristic opioid responses in mice: (a) antinociception; (b) respiratory depression; and (c) cataractogenic activity. We attempted to discover whether their apparent  $PA_2$  values could be differentially altered by pretreatment with an opioid with respect to dosage required and duration of effect. Significant differences were indeed found suggesting individuality of the receptors.

#### METHODS AND MATERIALS

Antinociception was measured using the hot plate method of Eddy and Leimbach (16). The hot plate was maintained at 55°C in a water bath. Respiratory depression was estimated indirectly by the rise in capillary blood  $pCO_2$  obtained from an incision in the ventral surface of the mouse tail near the proximal third. This capillary blood had a high  $pO_2$  of 80-100 millimeters, suggesting much arterial blood with little venous mixture. In order to reduce spontaneous clotting after

making an incision, the mouse was administered 100 units of heparin five minutes before the incision was made. Blood was collected without exposure to air using heparinized capillary tubes. The  $pCO_2$  and pH measurements were made with the aid of a Radiometer BMS-3. A 50% increase in respiratory depression was defined as three standard deviations above the mean  $pCO_2$  of capillary blood from untreated mice. This value is 33 mm, 7 mm above the mean  $pCO_2$  level in controls.

The  $AD_{50}$  (analgesic dose-50) was determined graphically by the method of Miller and Tainter (17). This quantal method required the use of at least thirty mice for each  $AD_{50}$  value. The  $ED_{50}$  for respiratory depression was also estimated graphically from at least four dose response curves using a minimum of fifteen mice for each curve. The  $ED_{50}$  for lenticular effect was measured according to the method of Weinstock (18) and the value obtained by the method of Litchfield and Wilcoxon (19).

Levorphanol tartrate was kindly provided by Hoffman La-Roche; naloxone hydrochloride was a gift from the Endo Company; and the methadone hydrochloride was given to us by Malinckrodt & Co. The dosages listed in the text are those of salt. Solutions were prepared in distilled water.

## RESULTS

### *EFFECT OF RESERPINE ON ANALGESIC AND RESPIRATORY RESPONSES*

When reserpine, 2 mg/kg, was injected twenty-four hours prior to hot plate testing using levorphanol, a large shift of the dose response curve was observed. The  $AD_{50}$  value for levorphanol rose from 7.5 mg/kg to more than 15 mg/kg whereas no change in the  $ED_{50}$  for respiratory depression was found as measured by the rise in the capillary blood  $pCO_2$ . These observations clearly indicate differences at least in modulating mechanisms.

### *THE $PA_2$ FOR ANTINOCICEPTION AND RESPIRATORY DEPRESSION*

Levorphanol and naloxone were injected subcutaneously thirty minutes prior to testing. The naloxone was injected on the contralateral side. The apparent antinociceptive  $PA_2$  was found to be 7.0 whereas the respiratory  $PA_2$  was 6.99. These values for naloxone-opioid are virtually identical and indicate apparently similar affinity constants for the two response mechanisms. Studies of the catalectic effect, using methadone as the opioid and naloxone as the antagonist, revealed a  $PA_2$  of 7.03 identical with that of respiratory and analgesic results. Thus it would appear that each of the three test modalities reveal receptor similarity.

While all of the systems which have been tested with morphine-like agonists show identical  $pa_2$ , Takemori et al. (20) have recently reported a higher  $pa_2$  (7.4) for respiratory depression. These investigators suggest that the respiratory opiate receptor differs qualitatively from the analgesic receptor. This difference in findings has not as yet been reconciled.

#### *EFFECTS OF SINGLE TREATMENT WITH LEVORPHANOL ON THE $ED_{50}$ OF THE THREE MODALITIES*

The preceding findings indicate no differences in receptor affinities. Studies were therefore undertaken to discover what change might occur in affinity, or in tolerance development, after opioid treatment. A single dose of levorphanol, 2 mg/kg s.c., given two days prior to testing, produced a significant ( $p < 0.05$ ) increase in the antinociceptive  $ED_{50}$ , from 0.34 mg/kg to 0.58 mg/kg. These results indicate that this small dose of levorphanol had induced tolerance lasting at least two days.

When mice were pretreated with levorphanol in a dosage of 45 mg/kg three days prior to testing, the respiratory  $ED_{50}$  remained unchanged (7.0 mg/kg vs. 6.98 mg/kg). These  $ED_{50}$  values are considerably greater than the  $ED_{50}$  for antinociception. However, the injection of levorphanol in a dosage of 15 mg/kg s.c. given four days before testing, increased the cartaractogenic  $ED_{50}$  from 14 mg/kg to 21 mg/kg. Lesser dosages did not produce measureable tolerance while larger single dosages induced more profound tolerance.

#### *CHANGE IN $pa_2$ WITH PRETREATMENT*

As shown earlier, the apparent  $pa_2$  for antinociceptive, respiratory depression or lenticular responses are similar. However, when the  $pa_2$  was determined two days after a single injection of levorphanol, 2 mg/kg s.c., a four-fold increase was obtained. The  $pa_2$  rose to 7.56. A similar increase was noted in the  $pa_2$  for respiratory depression but a larger dosage was required. Pretreatment, three days earlier in dosages of 10 to 40 mg/kg, increased the  $pa_2$  to 7.8 which is considerably greater than the mean value in controls of 6.99. Despite development of tolerance, the lenticular  $pa_2$  failed to rise when levorphanol was given four days earlier in a dosage of 15 mg/kg. The  $pa_2$  value was 6.9. This finding is of considerable interest because it indicates that no change occurred at the receptor site. Evidently tolerance to the lenticular effect develops at a site remote from the narcotic receptor initiating the event.



### *PRETREATMENT DOSAGE AND RELATION TO CHANGE IN $pA_2$*

Levorphanol, in a dosage of 2 mg/kg, increased antinociceptive  $pA_2$  from 7.0 to 7.6. Doubling the levorphanol dosage to 4 mg/kg, produced no further change, whereas decreasing the dosage to 1 mg/kg, modestly elevated the  $pA_2$  to 7.7. Halving this pretreatment dosage caused a substantial drop in  $pA_2$  to 7.4. These findings indicate that the change in receptor conformation occurs over a very narrow range of opioid dosage and is not increased further by raising the dosage even as much as four-fold. Very similar findings were seen with respect to respiratory depression: a dosage of 45 mg/kg injected three days earlier, raised the  $pA_2$  from 7 to 7.8 and a similar value was obtained using the dosage of only 10 mg/kg. Below this dosage a sharp falloff in  $pA_2$  to 7.4 was seen, and at the dosage of 4 mg/kg, the  $pA_2$  returned to about 7, indicating a very narrow dose relationship. The lenticular parameter was not further studied because no  $pA_2$  shift was observed in animals treated with 15 mg/kg, a dosage which produced a significant elevation in the  $ED_{50}$ .

### *DIFFERENCES AMONG THE THREE NARCOTIC RECEPTORS*

There is evidently a 10:1 difference in the dosage required to increase the  $pA_2$ , or initiate a pharmacological response of the respiratory receptor, as compared to actions on the antinociceptive receptor. An additional difference between the respiratory and analgesic receptor mechanisms concerns the duration of the  $pA_2$  elevation for the respiratory response, as opposed to the antinociceptive center. The former remains elevated for up to nine days. A similar study by Takemori et al. (12) indicates that the  $pA_2$  for the analgesic time course of morphine persists for only four days. Whether this is related to the initial dosage is not yet known.

### *DISCUSSION*

More than two decades ago, Schneider (21) showed that treatment with reserpine attenuated the antinociceptive response to morphine in mice. Others (22-25) have recently shown that stimulation of dopaminergic receptors, or inhibition of serotonin synthesis leads to inhibition of morphine analgesia in mice. Cholinergic systems also are proposed as positive modulators of morphine analgesia. In our previous study we found that reserpine had no effect on the respiratory response to levorphanol, indicating that this response differed significantly from the antinociceptive response. Present studies revealed a further difference:

tolerance develops to the antinociceptive action, but not to the respiratory depressive effect of levorphanol after a single dose, despite the fact that the antinociceptive and respiratory responses show the same  $PA_2$ . The indifference to biogenic amine modulation shown by the respiratory receptor mechanism, and the failure to develop tolerance, strongly suggests that respiratory and analgesic responses are mediated by different receptors.

In an earlier study, Takemori et al. (13) found that pretreatment of mice with morphine increased the apparent  $PA_2$ . This finding, using levorphanol, applies not only to the analgesic response, but to the respiratory response as well, although the latter requires nearly ten times as large a dose to initiate receptor change. This requirement corresponds to the dosage ratio for respiratory depression as compared with antinociceptive action. This differential could result from reduced perfusion of the respiratory region, so that the concentration of levorphanol might be very different from the concentration found in the region mediating antinociception. Alternatively there may be lesser numbers of receptors for the antinociceptive response, thus fewer receptors may require a lower drug concentration. Neither of these explanations would affect the  $PA_2$  findings because these represent ratios of agonist to antagonist, and do not involve, ultimately, absolute concentrations.

Tolerance to the lenticular or cataractogenic effect induced by levorphanol has been known for more than a decade. This phenomenon is clearly related to protein synthesis, since inhibitors such as puromycin or actinomycin-D block development of lenticular tolerance (15). Studies (22) showed that increased sensitivity to naloxone could be prevented if mice were pretreated with cycloheximide, an inhibitor of protein synthesis, indicating that the rise in  $PA_2$  results from *de novo* production of protein. In mice tolerant to levorphanol's cataractogenic effect, it was surprising to note that the  $PA_2$  value obtained in these mice for naloxone was no different than for naive animals. Evidently, the explanation lies in the fact that, unlike respiratory or antinociceptive receptors, cataractogenic tolerance is unassociated with conformational change in the receptor that initiates transient lenticular cataracts.

Cataracts develop from profound sympathetic stimulation and can be attenuated by chemical sympathectomy with reserpine, or by treatment with adrenergic antagonists (23). It would appear that sympathetic stimulation, a hallmark of opioids but not their antagonists, differs remarkably from the analgesic and respiratory depressive responses, despite the fact that the initiating receptor possesses similar constituents as judged by similarities of  $PA_2$ .

The increase in  $PA_2$  which accompanies tolerance to the antinociceptive action, is narrowly related to the dosage of opioid used. A rise in  $PA_2$  is seen at dosages of less than 1 mg/kg but does not increase further with doses above 4 mg/kg. The fact that a rise in respiratory  $PA_2$  exhibits a similar narrow range, suggests that mechanisms for inducing conformational changes in the receptors are very similar, with the notable exception that a much larger dosage is required to produce respiratory receptor alteration. This differential in dosage is proportional roughly to the dosage required for antinociception vs. respiratory depression. Because no change took place at the site initiating the lenticular phenomenon, we conclude that this receptor site differs from the others. In short, the evidence is substantial that each of these three major effects produced by opioids, is initiated by receptors that have significant pharmacological differences. As yet, there is no evidence that they lie in different regions, although earlier studies (24) showed that the  $PA_2$  for analgesia was much larger when the levorphanol was injected i.p., whereas the  $PA_2$  for respiratory depression was nearly twenty-fold lower under the same circumstances. Naloxone was injected s.c. in each case. It may well be that the blood supply differs from one region to the other.

It has been suggested (6) that tolerance to the antinociceptive or other effects of opioids arises because of increases in endogenous morphine-like ligands. These ligands are variously known as endorphin or enkephalin. Increases in endorphin would decrease the available sites for opioid binding, thus requiring larger dosages of opioid to produce a given effect: hence tolerance. While this hypothesis is quite attractive, no tolerance developed to the respiratory depressant action of levorphanol, although there was a significant increase in  $PA_2$ , whereas tolerance did develop to the antinociceptive action along with a rise in  $PA_2$ . It seems unlikely that increases in endorphin should occur with one response and not the other. Furthermore, the  $PA_2$  was not altered for the lenticular opacity effect despite tolerance development. In conclusion, evidence has been presented which seems to distinguish three major receptor mechanisms. The evidence consists of the ability of two of the three responses to show tolerance to a single dose. But in one of these, the lenticular response, the  $PA_2$  does not increase, whereas, in a third, the increase in  $PA_2$  for the respiratory effect was not accompanied by tolerance development. Additionally there are substantial differences in sensitivity to changes in receptor conformation induced by pretreatment. An increase in the respiratory  $PA_2$  requires about ten times the initiating dosage as does a comparable rise in the antinociceptive  $PA_2$ . That there may be a larger number of respiratory receptors is

one explanation, but perhaps a better one is reduced access to this region as compared with the antinociceptive receptors. Our findings (24) that the  $pA_2$  for naloxone-levorphanol fell to 6.4 for respiration vs. 7.7 for antinociception when levorphanol was injected i.p. (and the naloxone s.c.), fits the hypothesis of different sites and different access. It should be mentioned that peak pharmacological effects were observed at the same test interval: thirty minutes after injections.

The notion that receptor sites may differ substantially is further supported by the studies of Akera et al. (25) concerning differential effects of sodium on two types of opiate binding sites. In this experiment  $Na^+$  stimulated the saturable naloxone binding in thalamus-hypothalamus regions, but inhibited it in cerebellum. The authors (25) conclude that their findings strongly support the hypothesis that two types of naloxone binding sites exist in brain tissues.

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## 19. PHARMACOLOGY OF ENDOGENOUS OPIATE-LIKE PEPTIDES\*

Horace H. Loh and Ping-Yee Law

Langley Porter Neuropsychiatric Institute and Department of Pharmacology, University of California, San Francisco, California, 94122.

### INTRODUCTION

The presence of endogenous substances which interact with existing cellular receptors has been observed in numerous systems. Steroids express their action by interacting with cytoplasmic receptors in the target tissue. Peptide hormones, e.g., insulin, express their action by interacting with their respective membrane receptors and thus altering the intracellular level of c-AMP. Since it was demonstrated by Terenius (1), Pert and Snyder (2), Simon et al. (3), and Wong and Horng (4) that analgesic action of the opiates probably was the consequence of a stereospecific interaction between the opiates and receptors in the CNS, isolation of an endogenous substance which has high affinity for such receptors is probable. The interaction between the opiates and the receptors is highly stereospecific, the receptors have functional specificity and only compounds with opiate agonistic or antagonistic properties have appreciable affinity for the receptor [neurotransmitters have no affinity (4,5)]. Intuitively, it is unlikely that there be a highly specific receptor with functional activity and no endogenous ligands to interact with. Such an endogenous substance was proposed initially to exist in the neural tissues by Collier (6) in 1972, and later by Goldstein (7) in 1973. Their postulation was not without experimental basis. Earlier reports by Murray and Miller (8) and Huidobro and Miranda (9) suggested the presence of an endogenous agonist-like substance. Murray and Miller reported the lowering of narcotic AD<sub>50</sub> with a pituitary extract of oxytocin which was absent when synthetic oxytocin was used instead. Huidobro observed the modification

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of morphine response in mice by extracts from mice chronically treated with morphine. More important, the intrinsic activity of naloxone in various systems further supports the postulation of the existence of an endogenous compound. It has been observed that naloxone (1) increases acetylcholine release in the *in vitro* preparation of guinea pig ileum (10); (2) enhances the nociceptive response in rats and mice (11); and (3) reverses electroanalgesia in rats (12). Such observations could be attributed to naloxone antagonizing the action of an endogenous morphine-like substance although Goldstein et al. (13-15) have found otherwise. It is the purpose of this chapter to review the results of the search for endogenous morphine-like substances, namely enkephalins,  $\alpha$ - and  $\beta$ -endorphin and other related peptides. We will also review the present evidence for the existence of an antagonist-like substance and the numerous peptides which modulate morphine effect, namely adrenocorticotrophic hormone (ACTH), melanocyte-stimulating hormone (MSH), thyrotropin-releasing hormone (TRH) and Substance P.

#### ENDOGENOUS SUBSTANCES WITH MORPHINE-LIKE ACTIVITY

Before the isolation of any substance, specific assay methods to distinguish such substances from others have to be available. Stereospecific binding of radioactive opiates to the synaptic plasma membrane (SPM) fractions as suggested by Goldstein et al. (16), and the bioassay of mouse vas deferens and the longitudinal muscle preparation of the guinea pig myenteric plexus as described by Kosterlitz et al. (17,18) have been the methods of choice for the *in vitro* assay of any morphine-like substance. In both cases, steps have to be taken to ensure that any observed inhibition in stereospecific binding, or in electrically stimulated contraction of mouse vas deferens and guinea pig ileum by any substance, is an opiate specific event. For the receptor binding assay, since the interaction between agonist and receptors is greatly affected by cations (19,20), inhibition in  $^3\text{H}$ -opiate binding to the membrane fractions by any morphine-like substance must be sensitive to  $\text{Na}^+$  concentration. In the case of bioassay, inhibition of the electrically stimulated contraction in the vas deferens or ileum, by any substance, has to be reversible, or blocked by, the opiate antagonist, naloxone, and not by other receptor blockers such as propranolol or hexamethonium. Only after any endogenous substance exhibits such properties in the binding assay and the bioassay can it be considered as an opiate-like substance.

#### Enkephalin



Enkephalin\* is the first endogenous substance isolated from mammalian brains which inhibits morphine-like activity *in vitro*. Hughes (21), Terenius and Wahlstrom (22,23), and later Pasternak *et al.* (24), independently reported the existence of a factor which inhibited the electrically stimulated contraction of mouse vas deferens and guinea pig ileum (21), and also inhibited  $^3\text{H}$ -dihydromorphine binding to the SPM (22-24). Although the methods of isolation were different in all three cases, and the molecular weight estimation varied among the three groups (800-1200), the properties of their factors were so similar that we consider that all three groups probably have the same compound: enkephalin. This proved to be the case, for recently Simantov and Snyder reported the isolation and purification of their factor from the bovine brain which turned out to have the same sequence as enkephalin (25).

#### *Localization*

A relatively high level of enkephalin is found in the brain (0.42-0.55  $\mu\text{g}$  of normorphine equivalence per gm. rat brain) (21). It is unevenly distributed in the brain and the pattern of distribution closely resembles the distribution of opiate receptors in the brain (26,27). The release or destruction of enkephalin could partially explain the increase in opiate binding in the membrane preparation after preincubation (28). Enkephalin is further suggested to be contained in the synaptosomal fraction by Pasternak *et al.* (24). They obtained their major activity in the P2 (crude mitochondria and nerve endings) fraction, and the activity was released into the supernatant when the material was lysed. These authors suggested that enkephalin may be a putative neurotransmitter in certain nerves. A morphine-like substance with enkephalin properties was found in human cerebrospinal fluid (CSF) by Terenius (29) and in a peripheral system which has morphine receptors, namely guinea pig ileum (Hughes, personal communication).

#### *Structure and Properties*

Enkephalin was purified from porcine brain by Hughes *et al.* (30,31) and from bovine brain by Simantov and Snyder (25). In both cases, using mass spectrometry and Edman degradation, enkephalin was determined to be a mixture of two pentapeptides

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\* We will use the name enkephalin throughout this chapter only for the peptides with amino acid sequence H-Tyr-Gly-Gly-Phe-Met-OH and H-Tyr-Gly-Gly-Phe-Leu-OH as suggested by J. Hughes.

with the amino acid sequence of H-Tyr-Gly-Gly-Phe-Met-OH (Met-enkephalin) and H-Tyr-Gly-Gly-Phe-Leu-OH (Leu-enkephalin). In porcine brain, the ratio of Met-enkephalin to Leu-enkephalin is 4:1. The ratio is reversed in the bovine brain. The sequence provides the important tyramine moiety and the  $\beta$ -bend to fit the opiate receptors as suggested by Goldstein et al. (32), Bradbury et al. (33) and Horn and Rodgers (34). As a matter of fact, the primary sequence of enkephalin bears a remarkable resemblance to the heptapeptide synthesized by Goldstein et al. (32) which has slight opiate activity in the *in vitro* assays (H-Tyr-Gly-Gly-Gly-Lys-Met-Gly-OH). Molecular weight derived from this structure is significantly lower than the ones reported (640 vs. 800-1200). Such a discrepancy arose because of the interaction of enkephalin with the column materials as demonstrated by Simantov and Snyder with a Bio-gel column (25).

Enkephalin was found to be a very stable peptide. In aqueous solution at pH 5-6, it retained its activity for several months at  $-20^{\circ}\text{C}$  (21). It could be heated to  $100^{\circ}\text{C}$  for sixty minutes at neutral pH without loss of activity (25). But enkephalin is very sensitive to proteolytic enzymes (21, 24). The ability to inhibit ileum contractions and opiate binding could be destroyed by carboxypeptidase A and leucine-aminopeptidase (21,24). It is not sensitive to trypsin or neuraminidase. Whether the peptide is sensitive to chymotrypsin is not well resolved. Hughes reported enkephalin was not sensitive to chymotrypsin treatment (21), but Pasternak et al. reported the binding activity of enkephalin was affected by the enzyme (24). It is possible that the difference could result from the amount of enzyme used in the two studies. Since Hughes used 10  $\mu\text{g/ml}$  vs. 1.0  $\text{mg/ml}$  for Pasternak et al., a small contamination of exopeptidase in the chymotrypsin preparation would be magnified at the high concentration of enzyme and not at the lower.

### *Biological and Pharmacological Properties*

Both Met-enkephalin and Leu-enkephalin possess potent agonist-like activity *in vitro*. They produced a dose-related inhibition of electrocally stimulated contractions of mouse vas deferens and guinea pig ileum (21). This inhibition could only be stereospecifically reversed or blocked by opiate antagonists (30). Enkephalin is selectively active in the tissues known to possess opiate receptors. Met-enkephalin was reported to be twenty times more potent than normorphine in mouse vas deferens and equipotent with normorphine in guinea pig ileum (31). Cox et al. (35) reported that synthetic Met-enkephalin was only one-third as potent as normorphine in the ileum preparations. Data obtained in

our laboratory agree with Cox et al. Nevertheless, the difference in the potency in the two bioassays reported by Hughes et al. (31) is striking. The authors attributed the variation to the different amount of proteolytic enzyme activity present in the preparations. But, with the reported difference in  $PA_2$  value of naloxone to reverse enkephalin and normorphine ( $7.89 \pm 0.06$  and  $8.60 \pm 0.06$ , respectively) (30), and the stability of the preparation in the incubation mixture for up to five minutes (21), a difference in the potency between mouse vas deferens and guinea pig ileum could result from the dissimilar effect of the peptides on the adrenergic and cholinergic neurons. Though no tachyphylaxis could be produced by repeated administration of the peptides to mouse vas deferens or the guinea pig ileum, it is possible to demonstrate a cross tolerance between morphine and Met-enkephalin (36). The  $ID_{50}$  of Met-enkephalin increased 3- to 400-fold in the guinea pig ileum preparation from animals implanted with two or four morphine pellets for three days (36). This argues strongly that the site of action of enkephalin is similar to that of morphine, as suggested by theoretical calculations reported by Goldstein et al. (32) and Horn and Rodgers (34).

Enkephalin also shows activity in competing for opiate binding in SPM (22,23) or membrane preparations of rat (21, 22-25), or guinea pig brain (21,23,35). Double reciprocal analysis of the inhibition indicated that it is competitive (23). Binding of the peptide is further demonstrated to be at or near the opiate binding sites by the inhibition of the peptide activity with membranes pretreated with trypsin, chymotrypsin and sulfhydryl reagents (24). Stereospecific binding of opiates was shown to be affected by such reagents (28). Furthermore, the inhibition of  $^3H$ -naloxone binding by enkephalin is influenced by the concentration of cations (23-25, 37). The  $IC_{50}$  for Met-enkephalin increased from 8 nM to 30 nM in the presence of 100 mM  $Na^+$  (25). In the presence of 1.0 mM  $Mn^{++}$ , the  $IC_{50}$  decreased to 2.7 nM (25). Since enkephalin competed for  $^3H$ -morphine binding 40-fold better than  $^3H$ -naloxone binding (37), and the fact that  $Na^+$  decreased agonist binding and  $Mn^{++}$  enhanced agonist binding, enkephalin has definite agonistic properties in the *in vitro* binding system. With the exception of Hughes et al. (31), who reported that Met-enkephalin was three times more potent than normorphine in competing for opiate binding, all other reports (23,25,35,37) indicate that enkephalin is relatively less potent than normorphine. The value varied from 30% to 60% as potent.

Attempts to demonstrate any antinociceptive properties of enkephalin have been unsuccessful. Until recently, enkephalin administered centrally produced no effect in mice or rats. Only with the injection of 100 to 200  $\mu g$  of

Met-enkephalin intraventricularly, did enkephalin produce a slight increase in the latency of tail-flick response (38). The effect was observed by Belluzzi et al. to be transient (dissipated after ten to twelve minutes) and had a latency of two to six minutes after injection. The potency of enkephalin was much lower than that of morphine. Two hundred  $\mu$ g of Met-enkephalin produced an effect similar to 10  $\mu$ g of morphine sulfate (38). Similar results were obtained by Loh et al. (39) in determining the effect of Met-enkephalin in tail-flick, hotplate, and writhing tests in mice. Fifty  $\mu$ g of Met-enkephalin administered intracerebrally produced a weak analgesic response in all three tests, and the activity of enkephalin only lasted for five to ten minutes. Both groups were able to block the response to Met-enkephalin by pretreatment with naloxone. The transient effect of Met-enkephalin, and its low potency when compared with morphine sulfate *in vivo*, were generally believed to be due to the peptide sensitivity towards exopeptidases. Such an explanation might not be sufficient, since Wei and Loh (40) recently demonstrated development of opiate-like dependent behavior when rats were constantly infused in the periaqueductal grey region with a total of 15  $\mu$ g Met-enkephalin, or less, for seventy hours. All three signs of dependent behavior, wet shakes, teeth chattering and escaping phenomenon, were observed with the rats infused with Met-enkephalin. Rapid degradation of the peptides would be expected to eliminate any change in enkephalin level in the brain, and hence block the development of dependence on the peptide.

### Endorphins

The term endorphin, a combination of *endogenous* and *morphine*, was proposed by Eric Simon to signify any substance with opiate activity, such as the previously described substance, enkephalin. Indications of the existence of endorphins, other than enkephalin, are strong. Murray and Miller's report on the lowering of  $AD_{50}$  by a pituitary preparation of oxytocin was not observed in the hypophysectomized rats (8), thus suggesting the involvement of the pituitary glands. Moreover, there were numerous reports on pituitary peptides modulating the response to morphine [see review by George and Lomax (41)]. So the search for endorphin(s) present in the pituitary is on. The search is further prompted by the finding of Cox et al. (42) that a crude preparation of ACTH contained morphine-like activity which is absent in synthetic ACTH. [Goldstein's group named such a compound "pituitary opioid peptide-1 (POP-1)".] More recently, following structural studies of Met-enkephalin showing that its sequence is identical to residues 61-65 of  $\beta$ -lipotropin hormone

( $\beta$ -LPH), several peptides derived from the parent molecule,  $\beta$ -LPH, have been found to have opiate-like activity. Since two such peptides, " $\alpha$ - and  $\beta$ -endorphin" and POP-1 were all isolated from the pituitary, the probability that they were related to each other is high.

#### POP-1

Cox et al. (42) were the first to report the presence of any endogenous morphine activity in the extract of pituitary. They were able to demonstrate the activity with the guinea pig ileum assay in a crude preparation of ACTH (42), and in bovine pituitary extract (43). Their POP-1 activity in the ileum was reversible and blocked by naloxone. POP-1 has some properties similar to those of enkephalin. The substance is heat stable, shows activity in both the mouse vas deferens and guinea pig ileum preparation, and shows a positive  $\text{Na}^+$  effect in the competition binding assay with  $^3\text{H}$ -naloxone. But POP-1 differs from enkephalin in that its apparent molecular weight is larger, 1750, and its sensitivity toward various proteolytic enzymes is different from that of enkephalin. It is not sensitive to carboxypeptidase A and B, leucineaminopeptidase, ribonuclease, phospholipase A and B and phosphodiesterase. But POP-1 is sensitive to trypsin and chymotrypsin. It is possible that POP-1 is a molecule containing the pentapeptide sequence of enkephalin within its structure. Since the activity of the POP-1 was not completely destroyed by trypsin, there should be POP's other than those which have been isolated so far.

#### $\alpha$ - and $\beta$ -Endorphin

It was initially noted by Hughes et al. that the amino acid sequence of Met-enkephalin is present as residues 61-65 of  $\beta$ -LPH isolated from the pituitary glands of sheep (44), pig (45), camel (46), and man (47) (Figure 1). Hughes et al. thus suggested the possibility of  $\beta$ -LPH being the "pro-endorphin" in the pituitary. Since peptides with the pentapeptide sequence at the N-terminal had been isolated from the pituitary (48), and the same peptides could be obtained from  $\beta$ -LPH by mild digestion with trypsin *in vitro* (49), it was not unlikely that these peptides from  $\beta$ -LPH would have opiate activity.

The first such peptide was isolated by Guillemin et al. (50) from the porcine pituitary. Using the technique of high pressure liquid chromatography, Guillemin et al. were able to demonstrate opiate activity in a hexadecapeptide, termed  $\alpha$ -endorphin, with the bioassays and the binding assay. The peptide is 0.6 the potency of the normorphine in guinea pig

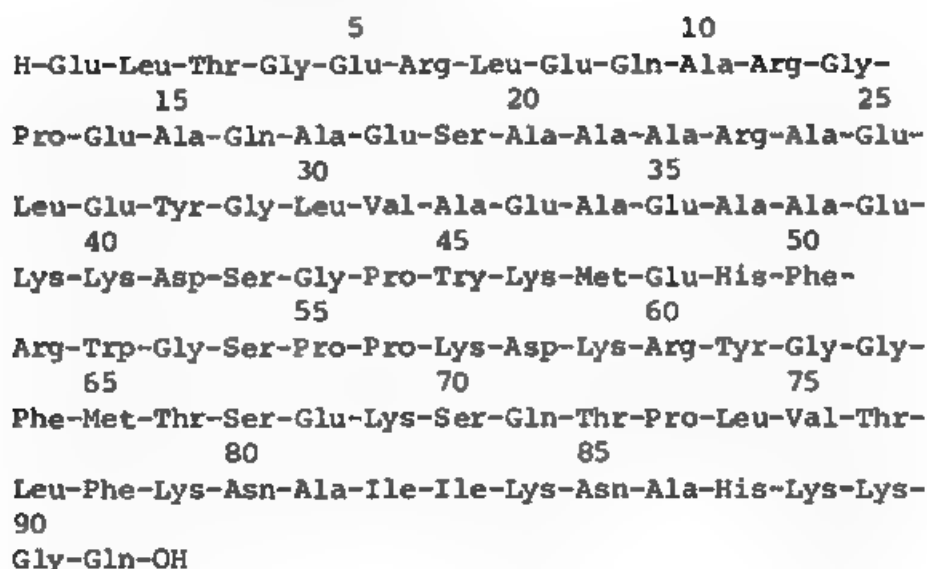


Fig. 1. The amino acid sequence of Ovine  $\beta$ -lipotropin ( $\beta$ -LPH) and functionally significant residues.

$\beta$ -MSH	residues 41-58
$\alpha$ -endorphin	residues 61-69
$\beta$ -endorphin [ $\beta$ -LPH-(61-91)]	residues 61-91
Enkephalin	residues 61-65

ileum preparation. After sequencing the peptide with mass spectrometry, the structure of  $\alpha$ -endorphin was observed to be identical to that of residues 61-76 of  $\beta$ -LPH. This further supported the original hypothesis that  $\beta$ -LPH may be the "pro-endorphin" molecule.

Concurrently, Li and Chung (46) reported the isolation of and untriakontapeptide named  $\beta$ -endorphin, with opiate activity from camel pituitary glands. The peptide sequence of  $\beta$ -endorphin was determined to be identical to those of residues 61-91 of  $\beta$ -LPH. In the binding assay,  $\beta$ -endorphin is three to five times more potent than normorphine in competing for  $^3\text{H}$ -opiate binding (46). It elicited a positive  $\text{Na}^+$  effect, although the effect was comparatively smaller in magnitude than that of Met-enkephalin (37). This might explain the lack of *in vivo* activity of Met-enkephalin and the potent antinociceptive response to  $\beta$ -endorphin (38,39). It is of interest that the parent molecule,  $\beta$ -LPH and peptides with N-terminal sequence other than the pentapeptide, e.g.,  $\beta$ -MSH (residues 41-58 of  $\beta$ -LPH), have no activity in the binding assay (35,37). When the activity of  $\beta$ -endorphin was determined with the guinea pig ileum preparation, it was found to be 0.4 times as potent as normorphine (35).  $\beta$ -MSH and  $\beta$ -LPH at concentration up to  $10^{-6}\text{M}$  were found to be inactive. Only at a very high concentration of  $\beta$ -LPH did Cox et al. (35) find some delayed activity.

By fractioning the ileum bath fluid with a Bio-gel P6 column, Cox et al. were able to demonstrate the appearance of binding activity at fractions with molecular weight less than 3000. Seemingly,  $\beta$ -LPH was converted to smaller active fragments. Indirectly, this further supported the importance of the pentapeptide sequence of Met-enkephalin at the N-terminal.

In a detailed study, Bradbury et al. (37) determined the sequences in  $\beta$ -LPH which have opiate-like activity in the binding assay. They found that the intermediate peptides of  $\beta$ -endorphin, peptides with the sequence corresponding to residues 61-68, 61-69, 61-87, 61-89 of  $\beta$ -LPH, all have agonist-like activity, i.e., they all compete for  $^3\text{H}$ -morphine binding better than  $^3\text{H}$ -naloxone binding and have a positive  $\text{Na}^+$  effect. No activity was observed with  $\beta$ -LPH,  $\gamma$ -LPH (1-58), N-fragment (1-38),  $\beta$ -MSH (41-58) or the 70-79 fragment. So all active fragments contain the pentapeptide sequence of Met-enkephalin at their N-terminal. There is a 30-fold difference in potency between  $\beta$ -endorphin and Met-enkephalin, 61-68, 61-69 and 61-87. Moreover, 61-89 is 20-fold more potent than 61-87, indicating the importance of the two lysine residues on 88 and 89 of  $\beta$ -endorphin. It is further demonstrated in the guinea pig ileum by Seidah et al. (51) that fragment 61-82 is equipotent to Met-enkephalin while 66-91 is completely devoid of activity.  $\beta$ -endorphin in their preparation is 10-fold more potent than Met-enkephalin. So with a small variation in the primary structure, there is a large difference in potency. The secondary structure and perhaps the tertiary structure probably have important roles in the *in vitro* and *in vivo* activity of  $\beta$ -endorphin.

The *in vivo* activity of  $\beta$ -endorphin has been demonstrated by Loh et al. (39) recently. It was found to be a potent antinociceptive agent when injected intracerebrally (39) (i.c.) and intravenously (52) (i.v.). When  $\beta$ -endorphin was administered intracerebrally into the mice, a naloxone reversible inhibition in the tail-flick, hot plate and writhing response was observed. This was found to be a dose dependent phenomenon and lasted for 60-90 minutes. On molar basis,  $\beta$ -endorphin is 18-33 times more potent than morphine sulfate (Table 1). Similar results were obtained with i.v. injections with the exception that  $\beta$ -endorphin is 3- to 4-fold more potent than morphine sulfate (52). It is not the conversion of  $\beta$ -endorphin to an active molecule which yielded the observed antinociceptive activity, since  $\beta$ -LPH was at least eighty times less potent than  $\beta$ -endorphin. Also,  $\alpha$ -endorphin, fragment 61-69 and the tryptic digest of  $\beta$ -endorphin were found to be inactive when 40  $\mu\text{g}$ , 20  $\mu\text{g}$  and 10  $\mu\text{g}$  per 25 g mouse was administered i.c., respectively. The fact that  $\beta$ -endorphin is acting like an opioid is substantiated by the cross tolerance and cross dependence studies. It was

demonstrated that the  $AD_{50}$  of  $\beta$ -endorphin increased with chronic treatment of mice with morphine (53). Furthermore, the withdrawal syndromes observed in morphine-dependent mice could be blocked by  $\beta$ -endorphin (53). Recently, Wei et al. have also found that constant infusion of  $\beta$ -endorphin produced dependent behavior in rats (40). Thus, all *in vivo* data indicate that  $\beta$ -endorphin mediates its effect via pathways similar to those of opiate analgesics.

TABLE 1

*$AD_{50}$  of  $\beta$ -Endorphin and Morphine in Mice\**

Tests	$\beta$ -Endorphin	Morphine Sulfate	Potency Ratio
Writhing	0.92(0.48-1.56)	17.92(10.76-27.52)	19.5
Hot Plate	2.32(1.4-3.96)	40.68(32.28-51.44)	17.5
Tail-Flick	1.52(0.88-2.64)	50.24(43.04-58.60)	33.0

\* $AD_{50}$  in nmol/kg i.c. (95% confidence limits)

The molecular weights of 3438 and 334 were used of  $\beta$ -endorphin and morphine sulfate, respectively.

#### Others

One possible candidate for being a morphine-like substance is bradykinin, a nonapeptide liberated upon treatment of plasma with trypsin or snake venom. When bradykinin was administered intraventricularly, increase in the threshold of the electrical stimuli applied to the tooth pulp of rabbit was observed (54,55). This antinociceptive activity of bradykinin was fast in onset (maximal ten minutes after injection) and is dissipated within sixty minutes. There was some functional specificity. Addition of amino acids to the N-terminal arginine of bradykinin decreased the antinociceptive potency even though the effects on vascular permeability and blood pressure increase (55). Although the activity of bradykinin was demonstrated not to be due to desensitization of the surrounding area of the tooth pulp, without the control experiments with the opiate antagonist, naloxone, bradykinin could not be considered to be an endorphin. Moreover, the affinity of bradykinin in the *in vitro* receptor binding assay was relatively low ( $IC_{50} > 10^{-5}M$ ) (56). Probably, the antinociceptive effect of bradykinin is caused by some physiological effect which bradykinin and morphine have in common, e.g.,



depletion of norepinephrine (57,58). Pretreatment with reserpine abolished morphine (59) and bradykinin (54) effects on the threshold of electric stimuli applied to the tooth pulp of rabbit.

Another candidate to be an endorphin is the morphine-like compound isolated by Levy et al. (60). Using an antibody specific for morphine, they were able to extract a morphine-like compound from the brain of rat, rabbit, cat and calf. This compound has a regional distribution similar to that of enkephalin, with the exception that this compound is present in the cerebellum. Chemical, chromatographic and immunological tests showed great similarities of the compound to morphine. But the compound is neither sensitive to any proteolytic enzymes nor sensitive to heating in 6 N HCl. More important, although this compound showed activity in the guinea pig ileum, the inhibition of the contraction was not reversed or blocked by naloxone. Thus, based on the criteria previously discussed, this compound is not an endorphin.

#### *ENDOGENOUS SUBSTANCE WITH ANTAGONIST-LIKE ACTIVITY*

Direct demonstration of the existence of an endogenous antagonist-like factor has not been successful. There are numerous reports supporting the hypothesis of the existence of such a factor. Reports such as the blockade of tolerance development to morphine by administration of protein synthesis inhibitors actinomycin D (71), puromycin (62) or cycloheximide (63) support the hypothesis. The numerous observations in the literature that ACTH and  $\beta$ -MSH antagonize morphine effects also indicate the existence of an endogenous antagonist-like substance (see review by Zimmerman and Krivoy on ACTH and  $\beta$ -MSH effect on morphine) (64). But so far, the identity of such a substance has not been clearly resolved.

One possible candidate for endogenous antagonist-like substance is the "tolerance factor" of Ungar. Ungar and Cohen (65) and later Ungar and Galvan (66) were able to demonstrate the transfer of tolerance with a chymotrypsin sensitive factor from brain extract of rats chronically treated with morphine to the naive animals. Intraperitoneal injection of such factor to the naive animal produced tolerance to morphine from three hours to ninety-six hours. Recently, Ungar (67) reported the purification of a factor and determined its structure to be H-Arg-Tyr-Gly-Gly-Phe-Met-OH. This structure has a molecular weight such that it might be the same compound which Terenius (68) reported to have antagonistic properties in the binding assay and could only be found in tolerant animals. But Ungar's *in vivo* observation could not be reproduced by Smith and Takemori (69). Furthermore, the method of administration (intraperitoneal) and the long duration in the

action of this factor make the significance of this observation unclear. Whether the hexapeptide, H-Arg-Tyr-Gly-Gly-Phe-Met-OH, is the endogenous antagonist-like substance could not be answered. For this hexapeptide has no antagonistic property in the mouse vas deferens (67) or the guinea pig ileum (70).

Other peptides which might be endogenous antagonist-like substances are ACTH and  $\beta$ -MSH. It was observed that  $\beta$ -MSH blocked the morphine inhibitory effect on the polysynaptic reflexes in cat spinal cord (71), that ACTH<sub>1-24</sub> antagonized the morphine effect on the isolate spinal cord (71,72) and the electric foot shock in rats (73), and that  $\beta$ -MSH release was stimulated by morphine (74). But the antagonistic activity of these two peptides might not be at the opiate receptor sites, since both peptides have low affinity for the opiate binding site (56,75). IC<sub>50</sub> for ACTH<sub>1-24</sub> was 3  $\mu$ M, comparatively higher than that of naloxone (2.4 nM) (37) and that of morphine (4.4 nM) (37). Moreover, the action of ACTH<sub>1-24</sub> most likely is mediated via glucocorticoids, for the antagonistic activity of ACTH<sub>1-24</sub> toward morphine was not observed in adrenalectomized animals (73,76). Only after supplementing such animals with dexamethasone could the effect of ACTH<sub>1-24</sub> be observed.

Another two peptides which modulate morphine's effect *in vivo* are TRH and Substance P. The distribution of TRH (77,78) and Substance P (79) in the brain suggested that these two peptides have important roles in synaptic function. When TRH was injected at the periaqueductal grey area (the site of morphine action) of rat brain, shaking behavior similar to that observed when naloxone was administered to dependent animals was observed (80). A crude preparation of Substance P antagonized the excitatory action of morphine on mice (81, 82). But this effect of Substance P was not observed with the purified preparation. Conversely, when synthetic Substance P was administered to mice, it abolished the abstinence syndrome (jumping) in morphinized mice (83). Without any activity in competing for opiate binding (55), the role of TRH and Substance P on morphine action, once again, might not be at the receptor level.

## DISCUSSION

In this chapter, we have reviewed the present evidence for the existence of any peptides which have opiate activity. As discussed earlier in the chapter, any substance which is considered to be endorphin must interact with the opiate receptors in the binding assay and should have activity in the bioassays which is reversible by or blocked by naloxone. The only peptides which satisfy both these requirements are

enkephalin,  $\alpha$ - and  $\beta$ -endorphin and a few  $\beta$ -LPH-related peptides. But the relative inactivity of enkephalin *in vivo* raises a question whether the *in vitro* assay methods yield a quantitative measurement for predicting analgesic potency for any endorphins. A previous explanation of the *in vivo* activity of enkephalin has been the degradation of the peptide by exopeptidases. Since the demonstration of the potent activity of  $\beta$ -endorphin *in vivo*, such an explanation might not be valid. Since the potency of  $\beta$ -endorphin to the binding assay is very sensitive to peptide length, any degradation by exopeptidases *in vivo* would diminish its activity. On the contrary,  $\beta$ -endorphin was found to be more active *in vivo* than *in vitro* (35,37,39,46). Moreover, the ability of a constant infusion of enkephalin to produce opiate-like dependent behavior in rats indicated that the level of enkephalin could be maintained at a higher than normal level in the brain. Since both *in vitro* methods are basically a measurement of receptor binding potential of the peptides, interactions between the "binding sites" (or regulation sites) and the "analgesic sites" (or catalytic sites) could not be measured. Probably, the secondary and tertiary structures of the peptide also play a vital role in the *in vivo* activity.

The demonstration that  $\beta$ -endorphin has *in vivo* antinociceptive activity further emphasized the role of  $\beta$ -LPH as the "prohormone" or the "pro-endorphin" in the pituitary. With the two existing peptides,  $\beta$ -MSH and  $\beta$ -endorphin having functional activity, it is not unlikely that more peptides derived from  $\beta$ -LPH would have functional activity. Since it has been well established that introduction of an allyl group will convert the opiate molecule from an agonist to an antagonist, it is attractive to postulate that with the introduction of other amino acids to the  $\beta$ -endorphin molecule, preferably at the N-terminal, the peptide would have antagonistic properties. Even though the hexapeptide proposed by Ungar has no antagonistic property (70,84), any addition of amino acid to that molecule might yield the long-sought endogenous antagonist-like substance. With  $\beta$ -MSH having some antagonistic properties, it might be of interest to investigate the peptides with the sequence of residues 41-91.

If such an antagonist-like factor exists, it might yield some insight into the problem of tolerance and dependence development. It has been suggested by Kosterlitz and Hughes (85) and more recently by Simantov and Snyder (86) that enkephalin is a neuromodulator or a putative neurotransmitter, controlling certain inhibitory mechanisms determining the rate of transmitter release. With a negative feedback mechanism on the synthesis of enkephalin, from the constantly stimulated receptor when opiates are introduced, tolerance will develop when more and more opiates are required to

maintain such an inhibitory mechanism or enkephalin biosynthesis. Such a postulation could not explain the recent observation by Simantov and Snyder (86) that the enkephalin level in the brain "increases" during tolerance development. We would like to postulate that the brain contains not only endorphin, it also contains an antagonist-like factor (ALF) which might have its origin from the  $\beta$ -LPH molecule. The endorphin(s) might regulate the inhibitory neurons and the ALF regulate the excitatory neurons. In the normal non-analgesic state, these two compounds would counteract each other. With the introduction of morphine, the equilibrium would shift to favor the inhibitory neurons and thus the analgesic state. In order to counteract the morphine effect the synthesis of ALF would increase. Together with the negative feedback of morphine on the synthesis of endorphin(s), the two phenomena would act synergistically to produce a high level of tolerance with a small change in the level of the two endogenous compounds. With the removal of morphine, the equilibrium would favor the excitatory neurons because of the rise of the ALF and the fall of the endorphin(s) level, thus resulting in the observed abstinence syndrome, jumping, teeth chattering and wet shakes.

In conclusion, the demonstration of the presence of endorphin is one of the most exciting findings in the field of narcotic research in recent years. Although the role of the peptides isolated so far in tolerance development is not known, we feel that a major step has been taken in the direction of solving the problem.

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## 20. REINFORCEMENT OF BEHAVIOR BY MORPHINE INJECTIONS\*

Steven R. Goldberg and Andrew H. Tang

Laboratory of Psychobiology, Department of Psychiatry, Harvard Medical School, Boston and New England Regional Primate Research Center, Southborough, Massachusetts and The Upjohn Company, Kalamazoo, Michigan.

### INTRODUCTION

Many drugs that affect the central nervous system can maintain behavior that leads to their injection; the injection thus functions as a reinforcer (1,2,3). As with any environmental event, the suitability of a drug injection to function as a reinforcer depends on a number of factors, including the antecedent behavior, the injected drug, and the scheduled temporal relations between them, as well as the history of drug administration. In most experiments with animals, behavior has been maintained under simple fixed-ratio schedules where every *n*th response produced a drug injection (2-6). The patterns of responding maintained under simple fixed-ratio schedules can be used as components of more complex second-order schedules of drug injection (5,7-9). Under a second-order schedule, a pattern of responding resulting from the operation of one schedule is treated as a unitary response that is itself maintained according to a second schedule. Second-order schedules of drug injection are of particular interest because they introduce a higher order of intermittency between drug injections and responses and allow evaluation of the roles of both drug injections and environmental stimuli associated with drug injections in the maintenance of drug-seeking behavior. These schedules are

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analogous in many ways to the complex schedules that control drug-seeking behavior in humans. With human addicts, intermittent injections of drug maintain the long sequence of behavioral activities involved in procurement, preparation and injection. Environmental stimuli associated with this behavioral sequence play an important role in the control of the behavior (10-12).

In one series of experiments, squirrel monkeys with permanent venous catheters responded under a second-order schedule where every thirtieth key press during a five-minute interval of time produced a two-second flash of a light (30-response fixed-ratio component); the first fixed-ratio component completed after the five-minute interval elapsed produced both the light and an intravenous injection of 100 µg/kg cocaine (7-9). This sequence was repeated fifteen times during each daily session. Although drug injections were highly intermittent relative to key presses, repeated sequences of rapid key pressing were maintained during each daily session. From 400 to 1000 responses preceded each injection of cocaine, and mean response rates exceeded one response per second in all monkeys. Also, the brief lights controlled patterns of responding characteristic of fixed-ratio schedules; a pause in responding after each light presentation was followed by an abrupt change to rapid responding until the light was produced again. The rates and patterns of responding controlled by the brief lights under this second-order schedule were similar to those controlled by intravenous injections of cocaine under simple fixed-ratio schedules where completion of every 30-response fixed-ratio component resulted in an injection of cocaine. When the brief lights were omitted but the frequency of cocaine injections was unchanged, rates of responding decreased and patterns of responding were disrupted. Similarly, when saline injections were substituted for cocaine injections, rates of responding decreased and patterns of responding were disrupted. Thus, both drug injections and stimuli associated with drug injections were important in the maintenance of the behavior. Under second-order schedules of intravenous cocaine injection with fixed-ratio components, high rates of responding can be consistently maintained throughout experimental sessions; moreover, there is strong moment-to-moment control of characteristic fixed-ratio patterns of responding.

High rates of responding and fixed-ratio patterns of responding also can be maintained under second-order schedules by intravenous injections of morphine (13-15). The effects of morphine under these schedule conditions are of particular interest because morphine has usually failed to maintain high rates of responding under simple fixed-ratio schedules (2,16). This chapter reviews experiments by Goldberg (13,14) and

Goldberg and Tang (15) that studied rhesus and squirrel monkeys under a second-order schedule in which intravenous injection of morphine occurred only at the end of each session and twenty-three hours or more elapsed between sessions, providing a separation between the reinforcing effects of morphine and its other pharmacological effects which may suppress behavior. Behavior of rhesus monkeys can be maintained under a similar second-order schedule by intramuscular injections of morphine (17). Since the route of administration may be a critical factor in using morphine to maintain behavior, this chapter also reviews these experiments.

## MATERIALS AND METHODS

### BEHAVIOR MAINTAINED BY INTRAMUSCULAR INJECTIONS OF MORPHINE IN RHESUS MONKEYS

Two male rhesus monkeys (*Macaca mulatta*) lived in primate cages enclosed in sound-attenuating isolation chambers. A response key and two 25-watt bulbs (white and red) were mounted on a transparent Lucite wall in the front wall of each primate cage. Each monkey wore a leather collar which was attached by a chain to the front of the cage. Once a day during initial training, the monkey's chain was drawn tight and his forearm pulled out through a small opening in the front of the cage; the experimenter then injected the monkey intramuscularly with 6 mg/kg of morphine. After several weeks, the monkey would immediately extend his arm out through the opening in the cage when the experimenter tapped on the cage.

Daily experimental sessions were subsequently conducted Monday through Friday using a second-order schedule of intramuscular morphine injection. A white light went on at the start of each session and every tenth key press during a five-minute interval of time produced a red light for two seconds, but had no other scheduled consequences (the 10-response fixed-ratio component of the second-order schedule; FR 10). The first FR 10 component completed after five minutes turned off the white light and produced a red light which remained on for two minutes while the chamber door was opened; in the presence of the red light, the monkey extended his arm and received an intramuscular injection of 6 mg/kg morphine (the five-minute fixed-interval component of the second-order schedule; FI 5 min). Once responding was well maintained, the interval was increased to a final value of sixty minutes. Using the nomenclature of Kelleher (18,19), the final schedule can be designated as FI 60 min (FR 10:S); that is, a visual stimulus (S) was briefly presented at the completion of each FR 10 component, and the first FR component completed after the 60-minute interval ended also resulted in injection of morphine.

After several months under the FI 60 min (FR (10:S) schedule of intramuscular morphine injection, the frequency of experimental sessions was reduced to three times a week, Monday, Wednesday and Friday. When response rates were stable, the dose of morphine per injection was decreased gradually from 6 mg/kg to 0 mg/kg (saline substitution). Each dose was studied for at least twelve sessions. For further details of these experiments see Goldberg et al. (17).

#### BEHAVIOR MAINTAINED BY INTRAVENOUS INJECTIONS OF MORPHINE IN RHESUS MONKEYS

Four male rhesus monkeys (*Macaca mulatta*) prepared with permanent venous catheters were kept in individual home cages with free access to food and water. During experimental sessions, each monkey was restrained in a Lucite chair and placed in a sound-attenuating isolation chamber. A response key and two 25 watt red bulbs were mounted on a transparent Lucite wall in front of the monkey; a 15-watt white bulb also was mounted on top of the chair. The monkey's venous catheter was connected by polyvinyl tubing to a motor-driven syringe located outside the isolation chamber. Operation of the motor-driven syringe was controlled by automatic programming equipment; duration of each injection was 200 msec and volume of each injection was 0.18 ml.

Daily experimental sessions were conducted Monday to Friday. Initially a white light went on at the start of each session and every third or 10th key press (FR 3 or FR 10) changed the light from white to red for two seconds and produced an intravenous injection of 0.1 mg/kg or 0.2 mg/kg morphine. Each session ended after about fifty injections or one hour. Once responding was maintained, the schedule was changed from the simple FR schedule to a second-order FI schedule with FR components. A white light went on at the start of each session and every FR 3 or FR 10 component completed during a 60-minute interval of time (FI 60 min) changed the light from white to red for two seconds; during brief presentations of the red light, responding had no scheduled consequences. The first FR component completed after sixty minutes turned off the white light and produced the red light which remained on during a series of five to ten injections spaced ten seconds apart. After the injections, all lights were turned off, and the monkey remained in the chamber for ten to fifteen minutes before being returned to its home cage. The total dose of morphine delivered at the end of each session was initially 1.0 mg/kg for monkeys AT and AW and 5.0 mg/kg for monkeys AR and AX. With these monkeys the FR requirement was increased gradually to thirty responses.

After performance of monkeys AT and AW stabilized under the FI 60 min (FR 30:S) schedule, saline was substituted for the 1.0 mg/kg dose of morphine for three (AT) or seven (AW) sessions, and responding declined to low rates. A low dose of 0.3 mg/kg (monkey AW) or 0.5 mg/kg (monkey AT) of morphine was then studied for eight to twelve sessions. With monkey AT, the schedule then was changed so that no stimulus change occurred at completion of each FR component; the red light was presented only in association with morphine injections at the end of each session. This schedule could be designated FI 60 min (FR 30). After twelve sessions with the brief light changes omitted, an additional four sessions were conducted with the brief light changes restored. Subsequently, the dose of morphine injected at the end of each session was increased to 5.0 mg/kg for ten sessions and the effects of omitting the brief light changes were again studied. For further details of these experiments see Goldberg and Tang (15).

#### *BEHAVIOR MAINTAINED BY INTRAVENOUS INJECTIONS OF MORPHINE IN SQUIRREL MONKEYS*

Four male squirrel monkeys (*Saimiri sciurea*) prepared with permanent venous catheters were kept in individual home cages with free access to food and water. During experimental sessions, each monkey was restrained in a Lucite chair and placed in a sound-attenuating isolation chamber. A response key and two green and two amber 6-watt bulbs were mounted on a transparent Lucite wall in front of the monkey. The catheter connections, motor-driven syringe system, and injection volume and duration were the same as those used with the rhesus monkeys.

Before the present experiments, the squirrel monkeys had been studied under various schedules of intravenous cocaine injection; training techniques were generally similar to those described by Goldberg (5). At the start of the present experiments, morphine injections were substituted for cocaine injections and the schedule of drug injection was changed to a second-order FI schedule with FR components. Each monkey was tested once a day, Monday to Friday. Initially, a green light went on at the start of the session and every 30th key press (FR 30) during a five-minute interval of time changed the light from green to amber for two seconds; during brief presentations of the amber light, responding had no scheduled consequences. The first FR 30 component completed after five minutes turned off the green light and produced fifteen consecutive injections of 0.1 mg/kg morphine; each 200 msec injection occurred at the onset of a two-second amber light. These light-injection pairings were spaced ten seconds apart so that over a period of 140 seconds the monkey received a

total dose of 1.5 mg/kg morphine. After the injections, all lights were turned off, and the monkey remained in the chamber for five to ten minutes before being returned to its home cage. Once responding was well maintained, the interval was increased to a final value of sixty minutes.

After performance stabilized under the FI 60 min (FR 30:S) schedule, the total dose of morphine injected at the end of each session was increased and decreased over a range of 0 (saline) to 6.0 mg/kg. Each dose was studied for ten to sixteen sessions. Subsequently, the morphine dose was returned to 1.5 mg/kg, and after ten sessions the schedule was changed so that no stimulus change occurred at completion of each FR component; two-second amber light presentations occurred only in association with morphine injections at the end of each session. After six sessions (monkey S-405) or fifteen sessions (monkey S-369) with the brief light changes omitted, an additional four to six sessions were conducted with the brief light changes restored. For further details of these experiments see Goldberg and Tang (15).

### Analysis of results

Mean rates of responding under the second-order schedules, with and without brief stimulus changes, were computed for each session by dividing total responses in the presence of the green or white light by total time the green or white light was present; responses during the two-second stimulus change to an amber or red light and total time the amber or red light was present were not included in computations.

### Drugs

Morphine sulfate was dissolved in saline (0.9% NaCl). All doses are expressed as the salt.

## RESULTS

### *BEHAVIOR MAINTAINED BY INTRAMUSCULAR INJECTIONS OF MORPHINE IN RHESUS MONKEYS*

Responding increased and then stabilized at a mean rate of approximately 0.10 response/second with monkey M-7 and 0.23 response/second with monkey M-9 under the final FI 60 min (FR 10:S) schedule of intramuscular morphine injection. A cumulative-response record, representative of final performance of monkey M-7 is shown in Figure 1. The briefly presented lights controlled patterns of responding characteristic of FR schedules (20); a pause in responding after each two-second red light was usually followed by an abrupt change



to rapid responding until the light was produced again. Pauses in responding were longest at the start of the 60-minute interval and became shorter as time elapsed in the interval. An overall pattern of positively-accelerated responding is characteristic of FI schedules (20). Monkey M-9 responded more rapidly than M-7, but there was occasional deterioration in the FR patterns of responding, and the positively-accelerated FI pattern of responding was less evident.

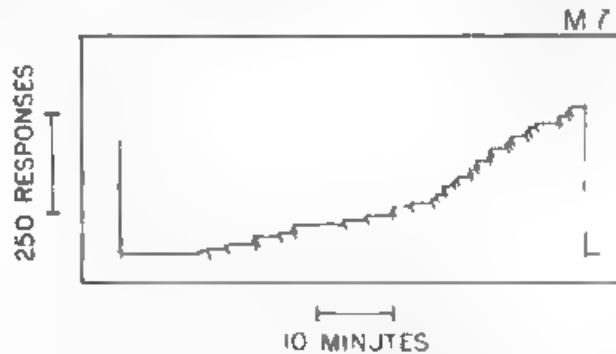
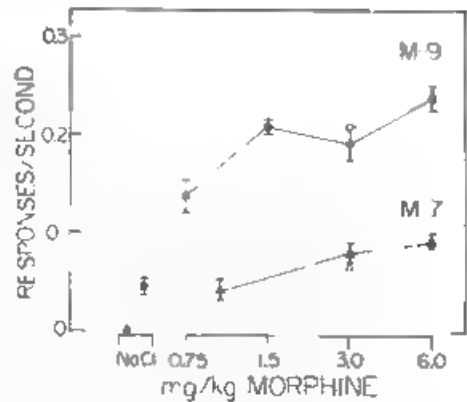


Fig. 1. Representative performance of rhesus monkey M-7 under the second-order schedule of intramuscular morphine injection. Ordinates, cumulative number of key-pressing responses; abscissae, time. Short downward deflections on the cumulative record indicate two-second presentations of a red light after every 10th response; during the two-second light presentations the recorder did not operate and responses had no scheduled consequences. The session ended with an intramuscular injection of 3 mg/kg morphine sulfate accompanied by a red light, which is indicated by the recording pen resetting to the bottom of the cumulative record. From Goldberg (14) with permission.

Mean response rates maintained by different doses of morphine or by saline are shown in Figure 2. Responding was well maintained by doses of morphine from 1.5 mg/kg to 6 mg/kg. Over this range there was no clear relationship between morphine dose and rates or patterns of responding. Before experimental sessions on Monday, when three days had elapsed since the last morphine injection, both monkeys often showed some signs of hyperactivity and hyperirritability, but a clear withdrawal syndrome was never observed. When the dose of morphine was below 1.5 mg/kg, responding decreased. When saline injections were substituted for injections of these low doses of morphine, responding of both monkeys further decreased and FR patterns of responding were lost. When 3 mg/kg injections of morphine were reinstated after twelve saline-substitution sessions, the previous rates and patterns of responding were restored within six sessions.

Fig. 2. Mean response rates of rhesus monkeys M-7 and M-9 under the second-order schedule of intramuscular morphine injection as a function of the dose of morphine sulfate injected at the end of the session. Ordinates: mean response rate; abscissae: dose. Each point represents the mean and the brackets the standard error from the last six sessions of saline (NaCl) substitution are shown by the open symbols. From Goldberg et al. (17) with permission.



#### BEHAVIOR MAINTAINED BY INTRAVENOUS INJECTIONS OF MORPHINE IN RHESUS MONKEYS

Rates and patterns of responding appeared stable after three to four weeks under the FI 60 min (FR 30:S) schedule of intravenous morphine injection. Cumulative-response records, representative of final performance of three monkeys at a dose of 5.0 mg/kg of morphine, are shown in Figure 3. As in the previous studies with intramuscular injections of morphine, characteristic FR patterns of responding were controlled by the briefly presented lights; a pause in responding after each two-second red light was followed by an abrupt change to rapid responding until the light was produced again. Also, the pauses in responding generally were longest at the start of the 60-minute interval and become shorter as time elapsed in the interval, a pattern of responding characteristic of FI schedules.

Although similar patterns of responding were maintained under second-order schedules by intramuscular and intravenous injections of morphine, much higher rates of responding were maintained by intravenous injections of morphine. At a dose of 5.0 mg/kg of morphine, given intravenously, mean response rates ranged from 0.4 to 0.7 response/second in the different monkeys. As shown in Figure 4, mean response rates as high as 1.0 response/second were maintained in two monkeys at 1.0 mg/kg morphine. When the dose of morphine was below 1.0 mg/kg, rates of responding decreased. When injections of saline were substituted for injections of morphine, rates of responding decreased further and FR patterns of responding were lost within five sessions. Previous rates of responding and characteristic FR patterns of responding could be restored within three sessions by reinstating injections of morphine. Although some signs of hyperactivity and hyperirritability were occasionally noted during saline substitution, a clear withdrawal

Fig. 3. Representative performances of rhesus monkeys AR, AT and AX under a second-order schedule of intravenous morphine injection. Ordinates, cumulative number of key-pressing responses; abscissae, time. Short downward deflections on the cumulative records indicate two-second presentations of a red light after every 30th response; during the two-second light presentations the recorder continued to operate but responses had no scheduled consequences. The recording pen reset to the bottom of the cumulative record whenever 1000 responses cumulated and when morphine was injected at the end of the session. The red light remained on at the end of the session until five or ten injections of morphine, spaced ten seconds apart, were delivered (a total dose of 5 mg/kg morphine sulfate).

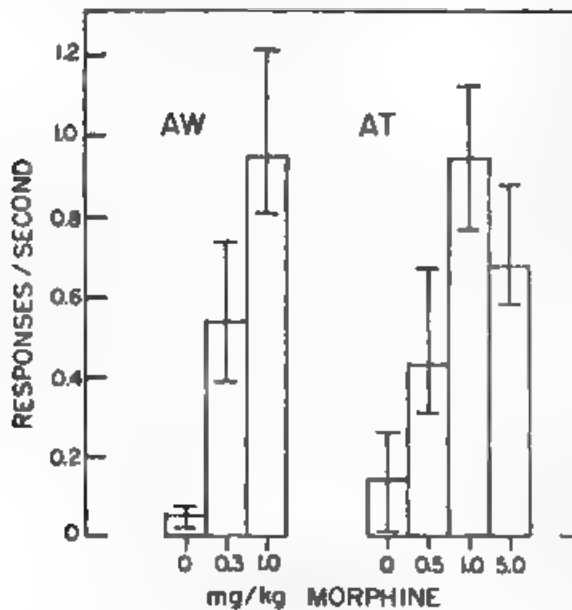
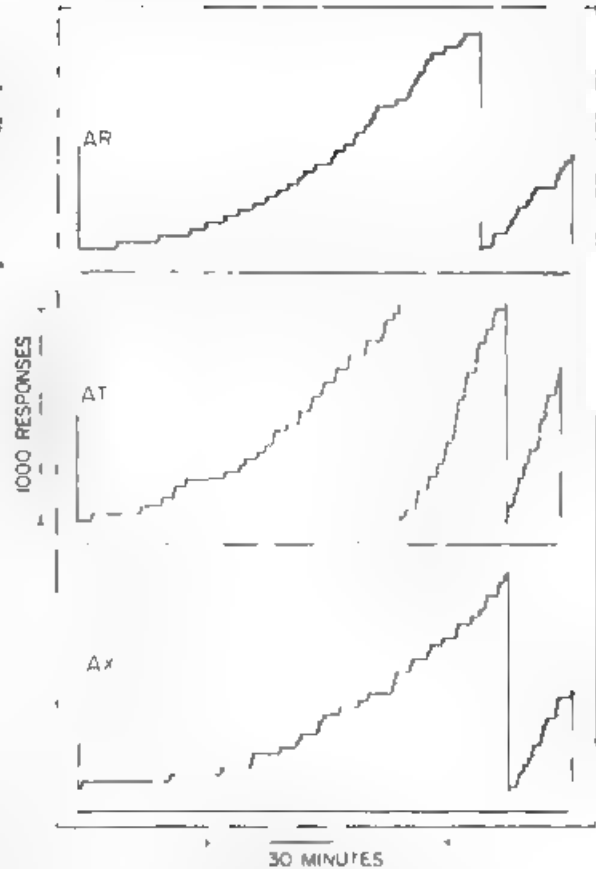


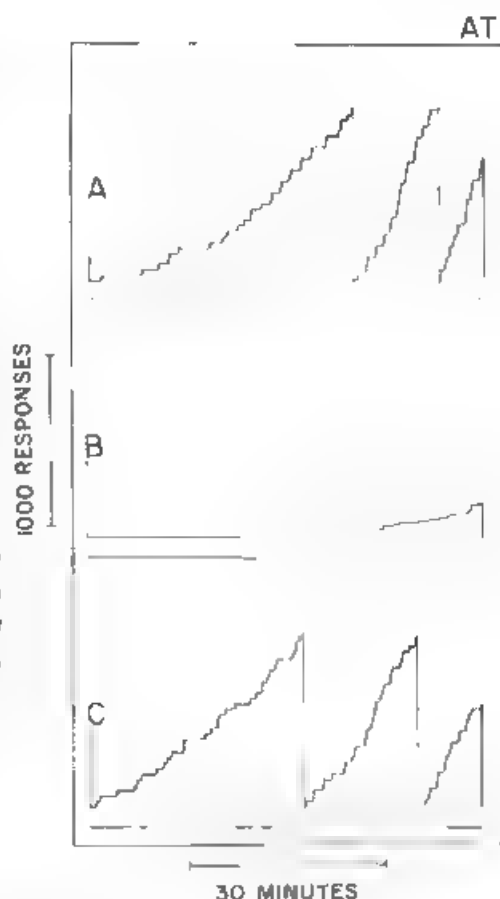
Fig. 4. Mean response rates of rhesus monkeys AW and AT under the second-order schedule of intravenous morphine injections as a function of the total dose of morphine sulfate injected at the end of the session. Ordinates: mean response rate; abscissae: dose. Each bar represents the mean and the brackets the range from the last five sessions at each dose of morphine and of the last two sessions of saline (0 mg/kg) substitution. Modified from Goldberg and Tang (15).

syndrome was never observed.

Cumulative-response records representative of the effects

of omitting the brief light changes with rhesus monkey AT are shown in Figure 5. A morphine dose as high as 5.0 mg/kg maintained mean rates of responding exceeding 1.0 response/second during the last thirty minutes of each session with monkey AT.

**Fig. 5.** Performance of rhesus monkey AT before, during and after omitting the brief light presentations under the second-order schedule of intravenous morphine injection. Recordings as in figure 3. Each record shows a complete session which ended with injection of a total dose of 5 mg/kg morphine sulfate. The records shown are from: the last session (A) before omitting the brief presentations of the red light; the 13th session (B) with no scheduled stimulus change at completion of each fixed-ratio component (the red light occurred only in association with morphine injections at the end of the session); and the second session (C) with the brief light presentations reinstated. Modified from Goldberg and Tang (15).



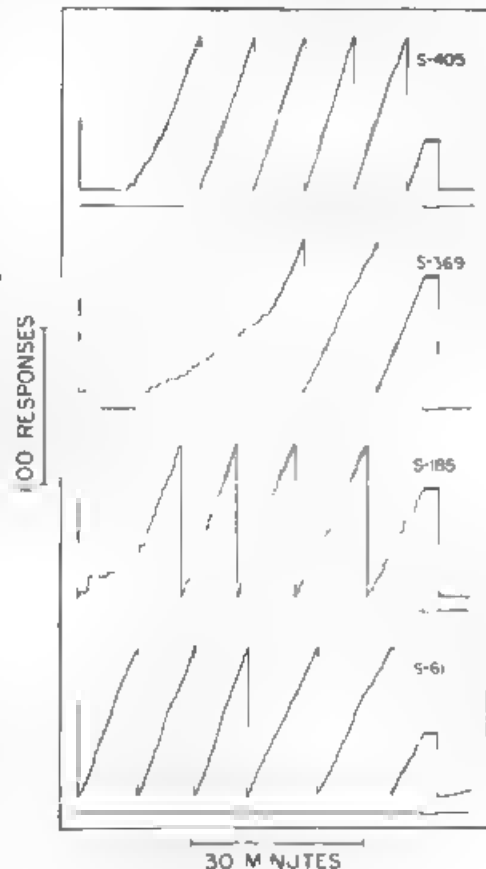
When the brief changes were omitted, but morphine was still injected at the end of each session, rates of responding decreased markedly in four to eight sessions and FR patterns of responding were lost. When the brief light change again occurred at completion of each FR component, the higher rates and characteristic FR patterns of responding were restored. When responding was maintained by 0.5 mg/kg of morphine with monkey AT, rates of responding fell to such low levels after six sessions with the brief light changes omitted, that during some sessions no responses occurred during the 60-minute interval (sufficient responding occurred several minutes after the end of the interval to produce injection of morphine and end the session). In order to restore responding when the brief light changes were reinstated at the 0.5 mg/kg dose of morphine, the FR response requirement was reduced to one and three during the first two sessions with the brief light changes reinstated. Responding increased during these sessions

during subsequent sessions, the FR response requirement was returned to thirty and higher rates of responding were again maintained by the 0.5 mg/kg dose of morphine.

#### BEHAVIOR MAINTAINED BY INTRAVENOUS INJECTIONS OF MORPHINE IN SQUIRREL MONKEYS

High rates and characteristic FR patterns of responding also were maintained under the FI 60 min (FR 30:S) schedule of intravenous morphine injection in squirrel monkeys. Figure 6 shows cumulative-response records representative of final performances of four squirrel monkeys at a dose of 1.5 mg/kg of morphine. High rates of responding were maintained throughout most of the session even though morphine was injected only at the end of the session; characteristic FR patterns of responding were controlled by the briefly presented lights.

Fig. 6. Representative performances of four squirrel monkeys under a second-order schedule of intravenous morphine injection. Ordinates, cumulative number of key-pressing responses; abscissae, time. Short downward deflections on the cumulative records indicate two-second presentations of an amber light after every 30th response; during the two-second light presentations the recorder continued to operate but responses had no scheduled consequences. The session ended with fifteen consecutive injections of 0.1 mg/kg morphine, spaced ten seconds apart (a total dose of 1.5 mg/kg morphine sulfate); each injection occurred at the onset of a two-second amber light. The downward deflection on the horizontal event line in each record indicates the period of morphine injection. The recording pen reset to the bottom of the cumulative record whenever 1100 responses cumulated and when the session ended.



For each of the squirrel monkeys, a mean rate of responding exceeding 1.0 response/second was maintained at a dose of 0.75 or 1.5 mg/kg of morphine. At lower doses of morphine, ranging from 0.3 to 0.75 mg/kg in different animals, mean

rates of responding were considerably lower but were still above saline-substitution levels. In squirrel monkey S-369, a dose of morphine as high as 6.0 mg/kg continued to maintain responding, although at somewhat reduced rates. When saline injection was substituted for injection of 1.5 mg/kg morphine, mean response rates decreased markedly and FR patterns of responding were lost within seven sessions, but rates and patterns of responding could be restored within a few sessions by reinstating injections of morphine. Withdrawal signs were never observed in squirrel monkeys when saline was substituted for morphine.

Representative cumulative-response records of monkeys S-369 and S-405 from sessions before, during and after six to fifteen consecutive sessions with the brief light changes omitted are shown in Figure 7. When the brief light changes were omitted, the resulting FI 60 min (FR 30) schedule closely resembled a simple 60-minute FI schedule of morphine injection. Although the same dose of 1.5 mg/kg morphine continued to be injected at the end of each session, omitting the brief light changes resulted in a marked decrease in mean response rates within two sessions. There was also a loss of the FR patterns of responding and the appearance of more pronounced progressively-accelerated patterns of responding. The final rates and patterns of responding under the FI 60 min (FR 30) schedule were similar to what would be expected under a long FI schedule (20). When a brief light change again occurred at completion of each FR component, the high mean rates and FR patterns of responding were immediately restored.

## DISCUSSION

Schedules of drug injection which restrict drug injection to the end of each session provide a means of studying the reinforcing effects of large doses of drugs such as morphine, which have pronounced suppressant effects on behavior. Pre-treatment with doses of morphine ranging from 0.3 to 5.6 mg/kg has been shown to markedly suppress responding maintained under FR schedules of intravenous codeine or cocaine injection in rhesus monkeys (2,21) and under FR or FI schedules of food presentation in rhesus monkeys (22,23) and squirrel monkeys (24,25). Under simple FR schedules of drug injection, where intravenous injection of morphine follows completion of each FR component and repeated injections can be obtained throughout and experimental session, doses of morphine above 0.1 mg/kg/injection have usually failed to maintain rates of responding above those maintained by saline injections (2,16). Under the present second-order schedules, however, morphine was injected only at the end of each session and doses of morphine ranging from 0.75 to 6 mg/kg functioned effectively

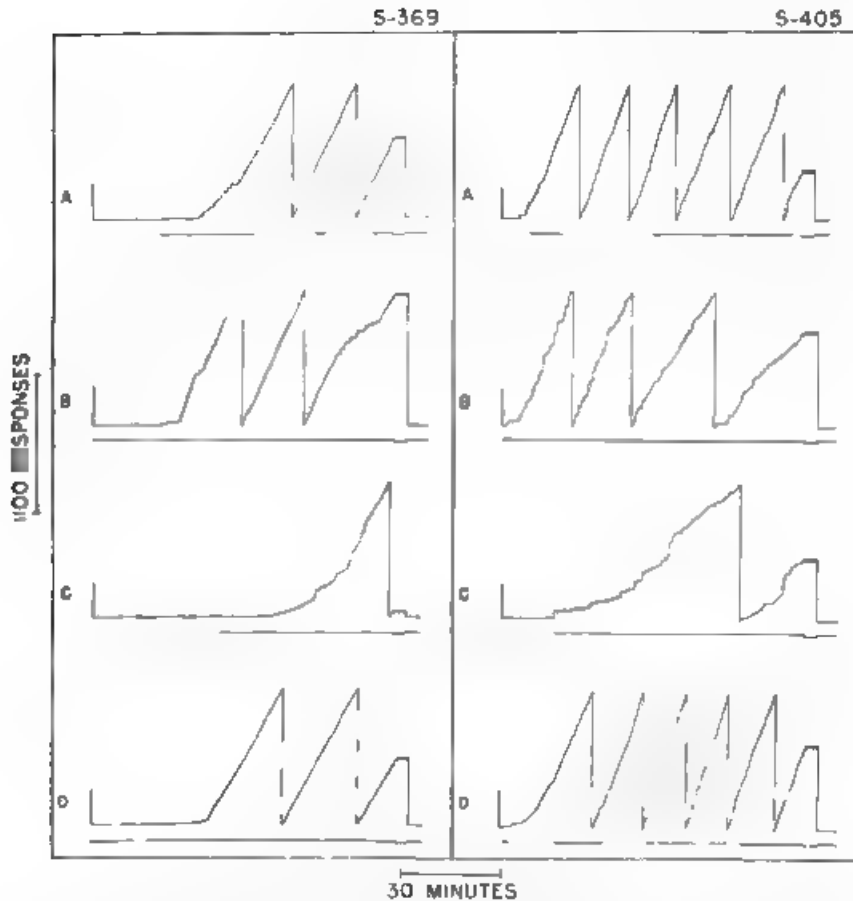


Fig. 7. Performances of squirrel monkeys S-369 and S-405 before, during and after omitting the brief light presentations under the second-order schedule of intravenous morphine injection. Recordings as in figure 6. Each record shows a complete session which ended with injection of a total dose of 1.5 mg/kg morphine sulfate. The records shown are from: the last session (A) before omitting the brief presentations of the amber light; the first session (B) and the last session (C) with no scheduled stimulus change at completion of each fixed-ratation component (the amber light occurred only in association with morphine injections at the end of the session); and the first session (D) with the brief light presentations reinstated. Modified from Goldberg and Tang (15).

as reinforcers and maintained rates of responding much higher than those maintained by saline injections. Schedules of drug injection in which the drug is injected only at the end of each experimental session allow one to study the reinforcing effects of a drug independently of its other effects on behavior.

A critical factor in using a drug to maintain behavior may be its route of administration. Intravenous injections seem particularly effective. In experimental animals prepared

with permanent venous catheters, drug can be injected immediately and without trauma after some response of the subject, and the effects of the drug usually occurs quickly. When responding was maintained by intravenous injections of morphine in the present experiments, final rates of responding were much higher than when responding was maintained under a similar condition by intramuscular injection of morphine. Although higher mean rates of responding were maintained by intravenous injections of morphine, intramuscular injections of morphine were able to consistently maintain responding over long periods of time and brief light changes associated with the injections modulated the control of the behavior.

Morphine is often presumed to be particularly effective as a reinforcer when it is given intravenously to a subject tolerant to large doses of morphine and currently in withdrawal. In the present experiments, however, intravenous injection of morphine maintained mean rates of responding exceeding one response per second even though the monkeys showed no clear signs of physiological dependence development. Other investigators have also demonstrated that intravenous injection of morphine can maintain responding in non-dependent rhesus monkeys independently of its efficacy in terminating the withdrawal syndrome (16,22,26,27).

In clinical situations of drug abuse and relapse to drug use, environmental cues and stimuli associated with the drug experience are believed to be contributory factors to such behaviors (10-12). The present second-order schedules of morphine injection provide a convenient experimental tool for determining the conditions under which environmental stimuli associated with extended sequences of drug-seeking behavior come to play an important role in the maintenance of and relapse to drug-seeking behavior. Under the present second-order schedules, drug was injected only at the end of the session and behavior during the session was controlled by intermittent changes in the color of the stimulus lights. The brief two-second light changes controlled local patterns of responding characteristic of FR schedules and appeared necessary for the maintenance of high mean rates of responding by intravenous injections of morphine. When the brief light changes were omitted during the session, but morphine continued to be injected intravenously at the end of the session mean rates of responding decreased markedly in both rhesus and squirrel monkeys. Further experiments are being conducted to determine the conditions necessary for brief environmental stimuli to facilitate drug-seeking behavior under second-order schedules. In the present experiments, for example, the light change always occurred during the series of morphine injections at the end of each session. Experiments are now being conducted to determine whether or not high rates and FR



patterns of responding can be maintained during the session if the brief light change follows completion of all but the final FR component and is never directly associated with morphine injection. This will allow assessment of the importance of temporally pairing environmental stimuli with the actual injection of drug.

#### SUMMARY

Under second-order schedules of intravenous morphine injections, high rates of responding were maintained when morphine was injected only at the end of each experimental session. During each session every 30th response produced a two-second light change. These briefly presented stimuli exerted important control over behavior as evidenced by a pause in responding after each light change followed by rapid responding until the light changed again, and by a marked decrement in rates of responding when the brief light changes were omitted during the session. Responding could be consistently maintained under a similar second-order schedule by intramuscular, rather than intravenous, injections of morphine, although rates of responding were lower.

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## 21. METABOLIC STEREOSPECIFICITY OF OPIATE AGONIST AND ANTAGONIST DRUGS

Steven H. Pollock and Kenneth Blum

Department of Pharmacology, Division of Drug and Alcohol Abuse, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, 78284.

### INTRODUCTION

Pharmacological activity of natural and synthetic opiates is stereospecific with respect to chemical configuration, i.e. (-)-enantiomers generally are biologically active, whereas, (+)-enantiomers usually tend to be less active or inactive even as antagonists. Metabolic stereospecificity also occurs and is of interest because it may provide insight into stereoselective mechanisms. Various pathways have been established in metabolism of opiates and some of these routes have been reviewed by Way and Adler (1) and later by Way (2). Recently pathways in metabolism of the narcotic antagonist naloxone (N-allyl-7,8-dihydro-14-hydroxy-normorphinone) have been delimited by Misra et al. (3) (Figure 1).

The pathway of N-dealkylation for which enantiomeric substrate stereoselectivity studies have been reported will be considered first for historical perspective, but attention will be focused primarily on 6-keto reduction of naloxone and other dihydromorphinone derivatives for which metabolic product stereoselectivity is operative producing  $\alpha$  and  $\beta$ -hydroxy diastereomers. Then other types of opiate metabolic stereospecificity will be considered.

### N-DEALKYLATION

N-dealkylation is a major metabolic route for naloxone in the rat (3) and also has been reported as a minor biotransformation pathway for this antagonist in man (4). Naloxone has not been subjected to a metabolic stereoselective comparison with its enantiomorph but studies have been conducted employing structurally related compounds. Initial findings that hepatic microsomal N-demethylating enzymes and opiate analgesic receptors are alike with respect not only to substrates and stereospecificity (5) but also to antagonism by

## Metabolism of Naloxone

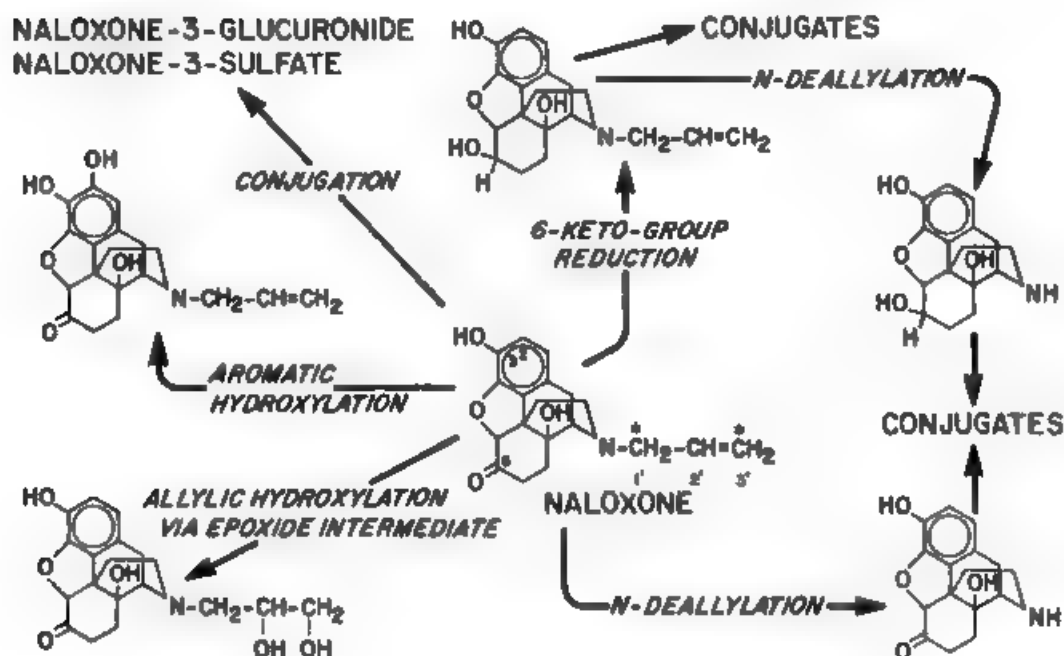


Figure 1

N-allylnormorphine (6) lead to the hypothesis that N-demethylation might serve as a mechanism in the development of tolerance to narcotic drugs (7). "The repeated administration of morphine [in vivo] reduced both enzymatic demethylation and pharmacological response" (7). Furthermore, in vivo administration of N-allylnormorphine not only blocked development of tolerance to morphine but also blocked reduction of N-demethylating activity (7). The tolerance theory proposed by Axelrod was soon doubted by Elison et al. (8) who observed on the basis of kinetic parameters (stable  $K_m$  but decreasing  $V_{max}$ ) that the narcotic N-demethylating system diminished in quantity without a change in enzyme quality. Attention was also focused on this pathway when oxidative N-dealkylation to produce nor-compounds was suggested by Beckett, Casey and Harper (9) "to be in the reaction sequence leading to analgesia." Nalorphine (N-allyl-normorphine) was soon reported, however, to be N-dealkylated at an even faster rate than morphine by male rat hepatic microsomes (10) and both nalorphine and morphine were determined to yield normorphine in brain of living rats (11).

Species differences have been detected in the enzymatic demethylation of narcotic drugs. Whereas rat liver enzyme preparations were found to demethylate morphine at a faster rate than observed for rabbit liver preparations, 1-methadone and meperidine were demethylated faster by rabbit than rat

hepatic preparations suggesting that more than one type of opiate N-dealkylating enzyme may occur (5). Furthermore, a disproportion in N- and O-demethylating capacities of different species with respect to various opiates has been observed and suggests that different enzymes may be responsible for the two types of demethylation (12).

A marked sex difference in opiate N-dealkylating ability has also been observed. Female rat liver enzyme preparations are less active than such preparations from male rat liver in N-demethylating morphine, l-methadone, and meperidine and sex hormonal regulation appears to be operative since "administration of estradiol to male rats results in a decrease in enzyme activity while treatment of female rats with testosterone enhances enzyme activity" (5).

N-dealkylation substrate stereoselectivity studies of various opiate enantiomers have been reviewed by Jenner and Testa (13) and from their review it indeed becomes readily apparent that there are many conflicting results, the interpretation of which "is impossible until a clear picture of the stereoselective processes emerges" (13). Elison et al. (8), for instance, found that inactive or less active dextro rotatory (+) isomers of various opiates were N-demethylated more slowly than active levo rotatory (-) isomers in agreement with findings by Axelrod (5). When Takemori and Mantering (12), however, examined methyl substituted 3-hydroxy-morphinans for N- and O-demethylation by male mouse and rat hepatic preparations, no stereoselectivity for N- and little for O-dealkylation was observed whereas a prominent substrate stereoselectivity for N,O-demethylation occurred (Table 1).

TABLE 1

*Demethylation of l and d-isomers of Morphinan-type Drugs*

Type of Demethylation	Compound	$\mu$ moles HCHO formed/g hepatic tissue			
		mouse		rat	
		minutes 15	120	minutes 15	120
N-	l-3-hydroxy-N-methylmorphinan (Dromoran)	0.58	0.64	0.28	0.40
N-	d-3-hydroxy-N-methylmorphinan (Dextrorphan)	0.50	0.58	0.28	0.32
O-	l-3-methoxymorphinan	0.80	0.92	0.68	0.96
O-	d-3-methoxymorphinan	0.62	0.86	0.48	0.76
N, O-	l-3-methoxy-N-methylmorphinan (Lavamethorphan)	2.06	2.60	2.16	2.98
N, O-	d-3-methoxy-N-methylmorphinan (Dextromethorphan)	1.46	1.82	1.02	1.64

[Data from Takemori and Mantering (12).]

**TABLE 2**  
*Inhibitory Effect of 1- and d-3-hydroxy-N-allyl-morphinan on the Demethylation of 1- and d-isomers of Morphinan-type Drugs by Livers of Mice*

Compound	$\mu\text{moles HCHO}$ found/g tissue per 19 min.	Percent Inhibition
1-3-hydroxy-N-methylmorphinan	0.58	
1-3-hydroxy-N-methylmorphinan + 1-3-hydroxy-N-allylmorphinan	0.36	38
1-3-hydroxy-N-methylmorphinan + d-3-hydroxy-N-allylmorphinan	0.36	38
d-3-hydroxy-N-methylmorphinan	0.54	
d-3-hydroxy-N-methylmorphinan + 1-3-hydroxy-N-allylmorphinan	0.32	41
d-3-hydroxy-N-methylmorphinan + d-3-hydroxy-N-allylmorphinan	0.30	44
1-3-methoxy-N-methylmorphinan	2.50	
1-3-methoxy-N-methylmorphinan + 1-3-hydroxy-N-allylmorphinan	1.66	34
1-3-methoxy-N-methylmorphinan + d-3-hydroxy-N-allylmorphinan	1.76	30
d-3-methoxy-N-methylmorphinan	1.72	
d-3-methoxy-N-methylmorphinan + 1-3-hydroxy-N-allylmorphinan	1.06	38
d-3-methoxy-N-methylmorphinan + d-3-hydroxy-N-allylmorphinan	1.06	38
(1 $\mu\text{mole allyl derivative}$ was employed to antagonize 4 $\mu\text{mole morphinan substrate}$ )		

[Data from Takemori and Mannering (12).]

Curiously, inhibition of N-, O-, and N,O-demethylation of methyl substituted 3-hydroxymorphinans by d- and l-enantiomers of 3-hydroxy-N-allylmorphinan failed to show stereospecificity (Table 2).

The lack of stereospecificity observed in the antagonism of demethylation of methyl substituted 3-hydroxymorphinans by (+)- and (-)-isomers of 3-hydroxy-N-allylmorphinan suggests a dissimilarity between receptors for demethylation and analgesia (12).

#### 6-KETO REDUCTION

6-Keto reduction had been observed to be an important metabolic pathway for naloxone in chicken hens, the major urinary metabolite identified as N-allyl-14-hydroxydihydro-normorphine (EN-2265) as a 3-glucuronide (14). The principal urinary metabolite of naloxone in male human volunteers and male rabbits was found by Fujimoto (14,15) to be naloxone-3-glucuronide. Weinstein *et al.* (4,16) using a more sensitive thin layer chromatography technique later detected what they believed to be EN-2265 in hydrolyzed human urine and in hydrolyzed glucuronides isolated from rabbit urine, thereby establishing 6-keto reduction as a minor metabolic pathway for naloxone (Narcan<sup>R</sup>) in mammals. Despite widespread clinical use, metabolic information on structurally related compounds such as the potent analgetics dihydromorphine (Dilaudid<sup>R</sup>), oxymorphone (Numorphan<sup>R</sup>), oxycodone (Percodan<sup>R</sup>) and the antitussive hydrocodone (Hycodan<sup>R</sup>, Hycomine<sup>R</sup>, Hycotuss<sup>R</sup>) (Figure 2) was not available.

From considerations of the chemical similarity of the double bond in the allyl group of naloxone and the cyclopropyl group in the N-substitute of naltrexone (17), it was surprising when a compound having a 6-isomorphine configuration was isolated as a major urinary metabolite of naltrexone in man (18) (Figure 3). Attention was thus focused on metabolic product stereoselectivity of opiates. The novel naltrexone metabolite was later confirmed to be N-cyclopropylmethyl-6-iso-7,8-dihydro-14-hydroxynormorphine (19) and soon this stereoselected metabolite (6 $\beta$ -naltrexol) was identified as an *in vivo* reduction product in guinea pig (20) and an *in vitro* metabolite produced by an hepatic preparation from male rabbit (21). The rabbit preparation similarly yielded 6 $\beta$ -naloxol from naloxone (21). GLC analysis indicated that 97% of the guinea pig naltrexone reduction product was 6 $\beta$ -naltrexol and 3% 6 $\alpha$ -naltrexol (20), but the formation of 6 $\alpha$ -naltrexol and 6 $\alpha$ -naloxol by rabbit preparations was doubted on the basis of Fourier transform computerized NMR spectroscopy (21).





*DIHYDROMORPHINONE KETONE REDUCTASES*

Prior to stereochemical studies of naloxone and naltrexone-6-ketone reduction, investigation was launched to characterize the enzymes responsible for naloxone reduction in chicken and rabbit. Pollock (22,23) found the greatest reduction to occur by NADPH dependent enzymes from hepatic cytosol and developed a sensitive radioassay. The standard assay mixture consisted of 7.9  $\mu$ mole glucose-6-phosphate, 0.25  $\mu$ mole NADP<sup>+</sup>, 2 units crystalline glucose-6-phosphate dehydrogenase, purified reductase, 1  $\mu$ mole naloxone as substrate, and 0.01  $\mu$ mole <sup>14</sup>C-naloxone as a tracer in a final volume of 1.0 ml 0.05M KH<sub>2</sub>PO<sub>4</sub>/NaOH pH 7.4 buffer. Analysis was performed by scintillation counting of excised TLC spots located with iodoplatinate spray reagent.

Pollock (22,23) was able to distinguish these enzymes from other keto reductases such as aromatic alpha-keto acid reductase (24), lactic dehydrogenase (25), liver alcohol dehydrogenase (26,27,28,29), the beta-ketoacyl-ACP reductase component of fatty acid synthetase (30,31,32), an enzyme designated AK-reductase (33), and an alpha, beta-unsaturated ketone reductase (34). Since dihydromorphinone and its derivatives naltrexone and EN-1655 (N-cyclobutylmethyl-14-hydroxynormorphinone) were also observed by TLC to undergo 6-keto reduction *in vitro*, the novel reductases from hepatic cytosol were designated dihydromorphinone ketone reductases (DMKR's) (22,23).

Cofactor regulation of DMKR's appears to be operative since NADP<sup>+</sup> was found to be inhibitory in both rabbit (Table 3) and chicken DMKR preparations (22). Lower activity is observed when NADPH is employed without an NADPH generating system. This phenomenon evidently results from accumulation of NADP<sup>+</sup>.

For chicken liver peak DMKR activity occurred in fractions salted-out between 50% and 65% ammonium sulfate saturation and for rabbit it occurred in fractions salted-out between 50% and 60% ammonium sulfate saturation. Prior to dialysis of ammonium sulfate salted-out fractions, DMKR activity was found to vary with protein concentration. A ceiling effect was observed with both the 100,000 x g supernatant and undialyzed salted-out fractions of chicken and rabbit liver (Figure 4).

Prior to the dialysis specific DMKR activity (nanomoles naloxone reduced/mg protein/min) was found to decrease as protein concentration was increased and incubation time was prolonged (Figure 5).

These findings suggest that the concentration of an inhibitor is increased along with increases of hepatic protein in the assay mixture. After dialysis of peak

TABLE 3

*Inhibitory Effect of NADP<sup>+</sup>*

Group		% Naloxone Reduction		
		Mean	SEM	N
1	0.25 $\mu$ mole NADP <sup>+</sup> with standard generating system	27.700	0.90	2
2	1.0 $\mu$ mole NADPH	21.333	0.64	6
3	1.0 $\mu$ mole NADPH plus 0.25 $\mu$ mole NADP <sup>+</sup>	18.700	0.47	3
ANOVA F Ratio = 26.26		Treatment df = 2	Error df = 10	P < .001

## Student Newman Keuls Test

Mean		Mean	Difference	LSR	P < 0.05
27.700	-	21.333	6.367	2.247	significant
21.333	-	18.700	2.633	2.595	significant
27.700	-	18.700	9.000	3.595	significant

*Dialyzed 50% 60% ammonium sulfate salted-out fraction of rabbit 100,000  $\times$  g hepatic supernatant was employed to reduce  $10^{-3}$  M naloxone in 10 minute assays employing three different cofactor conditions. Statistical analysis was by analysis of variance and a Student Newman Keuls Test (35)*

ammonium sulfate salted-out fractions specific activity was found to be independent of protein concentration for naloxone reduction up to 30% and 60% respectively for rabbit and chicken preparations in standard assays of ten minutes. Furthermore, the Michaelis-Menten constant ( $K_m$ ) of naloxone

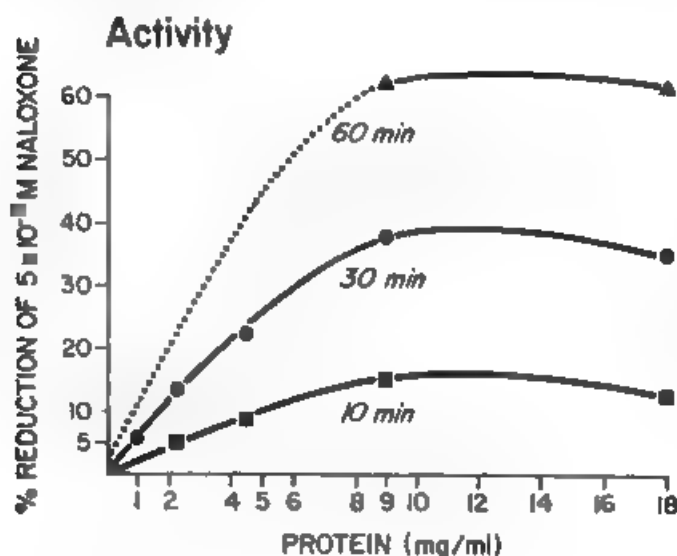


Fig. 4. Assays were performed using rabbit hepatic enzyme aliquots salted-out between 50% and 60% ammonium sulfate saturation.

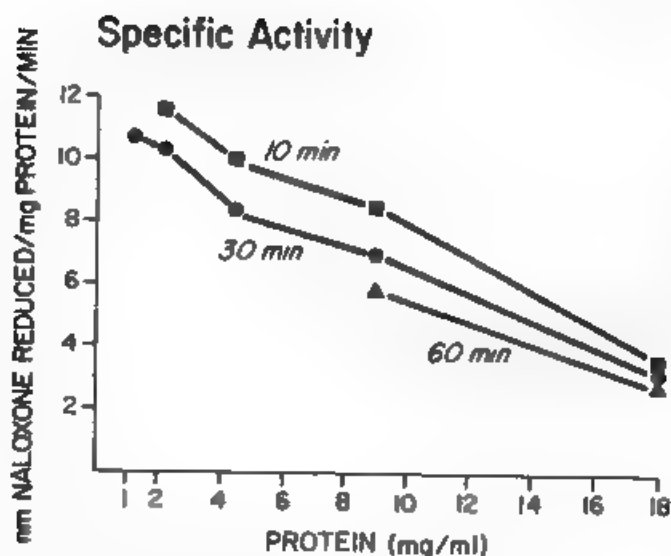


Fig. 5. Specific activities were determined using the same rabbit hepatic assays for which data were reported in Figure 4.

toward rabbit hepatic DMKR was measured at  $10^{-3}M$  using the 100,000 x g supernatant but was found to be in the range of  $10^{-4}M$  (once even measured at  $9.0 \times 10^{-5}M$ ) after dialysis of peak ammonium sulfate fractions.  $K_m$  findings for the chicken enzyme were similar, but specific activities of dialyzed peak ammonium sulfate salted-out fractions in standard assays tend to be in the range of 10-12 units for rabbit and 20 units for chicken hepatic DMKR's. These specific activities were not found to increase with increases in substrate concentration and thus represent measured  $V_{max}$ 's.

The pH optima for rabbit and chicken DMKR's were found to be approximately 7.4 and 7.0 respectively and the pH activity curves have a similar paraboloid shape. Molecular weights of rabbit and chicken DMKR's were determined to be in the range of 50,000 and 60,000 daltons respectively as estimated by G-100 Sephadex column chromatography and peak specific activities for eluates of nondialyzed salted-out enzyme fractions were in the range of 25 units for both rabbit and chicken preparations.

A direct comparison of reduction velocities of naloxone and naltrexone at  $10^{-3}M$  by rabbit and chicken preparations indicated no significant difference and reduction velocities were similar for the two substrates at concentrations near  $10^{-4}M$  using rabbit Sephadex enzyme eluate. Reduction velocities of naloxone and naltrexone were also similar when compared indirectly by using  $10^{-5}M$   $^{14}C$ -naloxone as tracer for each. Although for rabbit DMKR preparation hydromorphone behaved similarly to naloxone by indirect assay, EN-1655 is significantly different (Table 4). Hydromorphone and EN-1655 were found to be similar to naloxone by indirect assay with chicken DMKR preparation.

TABLE 4

*Indirect Comparison of Naloxone, Hydromorphone and EN-1655 as Substrates*

<u>10<sup>-3</sup> M Substrate</u>	<u>% Reduction of 10<sup>-5</sup> M <sup>14</sup>C-Naloxone</u>			
<i>Exp. 1</i>	<i>Mean</i>	<i>SEM</i>	<i>N</i>	<i>P</i>
Naloxone	21.0	1.14	4	
Hydromorphone	20.2	.42	4	N.S.
<i>Exp. 2</i>				
Naloxone	17.8	.27	4	
EN-1655	10.3	.32	5	< .001

*Experiments 1 and 2 were done on different days using dialyzed peak ammonium sulfate fractions of rabbit DMKR. Statistical analysis was done by a 2-tailed t test.*

Rabbit and chicken DMKR preparations were found to be different in their sensitivities to inhibitors relative to the isotope dilution effect of nonlabelled naloxone (Table 5).

The dissociative anesthetic ketamine (2-chlorophenyl-2-methylaminocyclohexanone) is a stronger inhibitor of chicken DMKR than of the rabbit DMKR system, whereas morphine is more inhibitory toward the rabbit enzyme system. Similarly dihydromorphone, nalbuphine (6 $\alpha$ -hydroxy reduction product of EN-1655), EN-2265 (6 $\alpha$ -naloxol), and EN-2260 (6 $\alpha$ -naltrexol) demonstrated very little product inhibition toward chicken DMKR but in comparison are strongly inhibitory toward the rabbit enzyme system (22,23).

After DMKR enzyme characterization studies were completed by Pollock (22,23), Roerig et al. (36) working in the same laboratory undertook a follow-up investigation of the same enzyme systems. Unfortunately, those investigators failed to account for impurities present in the <sup>14</sup>C-naloxone and <sup>3</sup>H-naltrexone which chromatographed as ca. 3% naloxol and 1% naltrexol respectively. The reliability of their enzyme activity and Km calculations is thus in serious doubt, especially when low velocities are involved. Not only did Roerig et al. employ nondialyzed ammonium sulfate salted-out protein fractions for which specific activities may have varied with their protein concentrations but they also failed to account for the substantial change in substrate concentrations which occurred during their thirty minute assays for Km calculations. It is thus no wonder that their Km values for naloxone were found to be almost an order of magnitude above those obtained by Pollock (22,23) for dialyzed enzyme preparations. While the kinetic data of Roerig et al. (36) are entirely unreliable, their inhibitor

TABLE 5

*Relative Inhibitory Effect of Various Compounds*

<u>Inhibitor</u>	<u>Relative Inhibitory Effect at <math>10^{-3}</math> M</u>	
	<u>Rabbit</u>	<u>Chicken</u>
Ketamine	0.8	1.2
Morphine	0.9	< 0.1
Hydromorphone	1.0	0.2
Nalbuphine	1.0	0.1
EN-2265	0.8	0.3
EN-2260	0.7	0.3
Naloxone-3-glucuronide	0.5	0.3
EN-2265-3-glucuronide	0.4	0.4
D-glucuronic acid	0.1	0.2
L-tyrosine	0.1	0.4

*The relative inhibitory effect at  $10^{-3}$  M was estimated by the following calculations:*

*Where,  $x$  = % reduction of  $10^{-5}$  M naloxone alone*

*$y$  = % reduction of  $10^{-5}$  M naloxone with  $10^{-3}$  M inhibitor present*

*$z$  = % reduction of  $10^{-5}$  M naloxone with  $10^{-3}$  M nonlabeled naloxone*

*% inhibition by inhibitor =  $100(x-y)/x$*

*% inhibition by isotope dilution effect =  $100(x-z)/x$*

*Therefore, relative inhibitory effect of inhibitor =  $(x-y)/(x-z)$*

[Data from Pollock (23)]

findings nevertheless are confirmatory and supplement results obtained by Pollock (22,23).

#### SOME STEREOSPECIFIC STUDIES OF DMKR ENZYME SYSTEMS

Assays of hepatic cytosol preparations had indicated a widespread species distribution of DMKR activity (22,23) and stereospecific studies of this activity were undertaken by Pollock and Dear (37) employing hydromorphone, naloxone and naltrexone as substrates. Additional substrates were reduced with rabbit and chicken DMKR preparations and evaluated by Pollock (38).

Naloxone·HCl, EN-2265 (6 $\alpha$ -naloxol), naltrexone·HCl, EN-2260A (6 $\alpha$ -naltrexol·HCl), Oxymorphone·HCl, EN-2370 (14-hydroxydihydromorphone), nalmexone·HCl (EN-1620A), EN-2261K (6 $\alpha$ -nalmexol), EN-1655A, nalbuphine·HCl, hydrocodone bitartrate, and oxycodone·HCl were provided gratis from Endo Laboratories, Inc. Hydromorphone·HCl was obtained from Knoll Pharmaceutical Company. Dihydromorphone, dihydrocodeine, 6 $\beta$ -naltrexol·HCl, and an additional sample of 6 $\alpha$ -naltrexol·HCl were supplied by the National Institute on Drug Abuse.

Methodology, findings, and observations follow.

### Enzyme Preparation

Fresh livers were obtained from animals sacrificed by decapitation except for calf and swine liver which was purchased from a local supermarket. Hepatic tissue was homogenized in a Waring blender using 0.106 M  $K_2HPO_4$ /0.07 M  $KHCO_3$  pH 7.9 buffer (2 ml/gram tissue). The crude homogenate was centrifuged for thirty minutes at 15,000 x g in a Sorvall Centrifuge. The supernatant was then centrifuged for ninety minutes in a Beckman Ultracentrifuge (Model L2-65) at  $R_{av} = 95,100 \times g$  ( $R_{max} = 131,000 \times g$ ). After removal of most of the surface lipid layer with a Kimwipes<sup>R</sup> tissue, the aqueous supernatant was transferred via Dispo-pipettes into a glass beaker. Swarz-Mann ultra pure special enzyme grade ammonium sulfate was used for enzyme salting-out, the quantities employed having been calculated from an equation based on saturation at 0°C (39). Fractions were collected by centrifugation at 15,000 x g for fifteen minutes. The desired fractions were resuspended in a minimal volume of dialysis buffer and dialyzed against one liter 0.005 M  $KH_2PO_4$ /NaOH buffer (pH 7.0) for twenty-four hours. All buffers employed in enzyme preparations were chilled to 4°C and an effort was made to keep tissue and enzyme preparations cold by working with an ice water bath or in a 4°C coldroom.

### Enzyme Assay

6-Carbinols of dihydromorphinones and dihydrocodeinones were produced in 250 ml Ehrlenmeyer flasks by incubation of  $6.0 \times 10^{-5}$  mole substrate with 240  $\mu$ mole glucose-6-phosphate (from Sigma), 7.5  $\mu$ mole  $NADP^+$  (from Sigma), 60 units crystalline glucose-6-phosphate dehydrogenase (Sigma type XV from Baker's yeast), and 3 ml dialyzed ammonium sulfate salted-out enzyme fraction in a final volume of 30 ml 0.05 M  $KH_2PO_4$ /NaOH buffer (pH 7.4) in a Dubnoff Metabolic Incubator at 60 oscillations/minute for two hours at 37°C. The assay mixtures were then decanted into glass stoppered bottles containing 30 ml 1.0 M  $NaHCO_3$ / $Na_2CO_3$  (pH 10.0) buffer and 60 ml ethyl acetate. After several minutes of vigorous shaking to extract metabolites into the organic phase, the phases were separated by centrifugation at 9,000 x g for five minutes. The ethyl acetate phase (top layer) was transferred by Dispo-pipette to a boiling flask and evaporated at 37°C under nitrogen.

Boiling flask residue containing metabolites and unutilized substrate was dissolved in 0.5 ml pyridine, 10  $\mu$ l was transferred to an acylation tube, and silylation was accomplished with addition of 10  $\mu$ l N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) from Analabs. The reaction was allowed to proceed for fifteen minutes. The silylation

mixture was evaporated under dried N<sub>2</sub> and the trimethylsilyl derivatives resuspended in 30  $\mu$ l ethyl acetate. Samples of 1.5  $\mu$ l were analyzed by gas-liquid chromatography employing a glass column (l = 2m, i.d. = 0.25mm, liquid phase = 3% OV-17 on GasChrom Q with 100/120 mesh) in a Varian GC (Series 1700) with a flame-ionization detector. Dried nitrogen (30 ml/min) was used as the carrier gas and hydrogen and air flows were 25 and 300 ml/min respectively. The column temperature was 255°C and the injector and detector temperatures were 265° and 275° respectively.

GLC-MS was obtained by integration of a Finnigan 1015 S/L Mass Spectrometer (Model 1015C) and a Varian GC (Series 1400). A Systems Industries System/150 data output device was employed to provide data printout. By GLC-MS the identity of derivatized standards and metabolites was established. Quantitation was based on peak height measurement. When 6-keto reduction was complete, metabolites were silylated to *bis*-TMS derivatives for this purpose. Since when reduction was incomplete a mixture of *bis* and *mono*-TMS derivatized metabolites was usually obtained, both derivatives were accounted for in quantitation.

### Findings

*Bis*- and *mono*-silylated derivatives of reduced dihydromorphinones having the 6 $\alpha$ -hydroxy configuration had shorter retention times than those having the 6 $\beta$ -hydroxy configuration (Figures 6,7). Trisilylated 6 $\beta$ -naloxol and 6 $\beta$ -naltrexol were ascertained to have shorter retention times than trisilylated 6 $\alpha$ -naloxol and 6 $\alpha$ -naltrexol respectively.

The 6-ketone moiety of naloxone was substantially reduced *in vitro* by hepatic preparations from most species studied (Table 6).

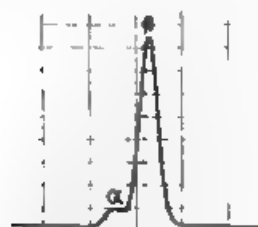
The 45-70% ammonium sulfate salted-out hepatic DMKR fraction of pigeon (White King, 1 M and 1F) and chicken (White Leghorn, 2 M and 2 F) produced only 6 $\alpha$ -naloxol as did a 0-45% fraction of DMKR from chicken (2 M). A 45-70% DMKR fraction from seven immature chickens (age between one and two weeks) resulted in complete naloxone reduction to 6 $\alpha$ -naloxol. Muscovy duck (1 M) preparation produced both 6 $\alpha$  and 6 $\beta$ -hydroxy diastereomers. Only the 6 $\beta$ -hydroxy epimer was detected after naloxone reduction by rat (Sprague-Dawley, 2 M) and swine 45-65% ammonium sulfate salted-out DMKR preparations, but the same fraction of calf, rhesus monkey (1 M), and New Zealand White Rabbit DMKR's produced some 6 $\alpha$ -naloxol in addition to 6 $\beta$ -naloxol. The ratio of 6 $\alpha$ -naloxol to 6 $\beta$ -naloxol was about 1:8 whether produced by the 45-60% fraction from male or female rabbit. The 0-45% fraction from the same rabbit sources yielded a similar predominance of 6 $\beta$ -naloxol. The 45-65%



fraction from two immature rabbits (age ten and thirteen weeks) was also active, completely reducing naloxone to 19% 6 $\alpha$ -naloxol and 81% 6 $\beta$ -naloxol. Furthermore, a 45-65% fraction

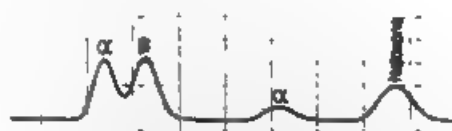
### Naloxone Metabolites

RABBIT (45-65 fraction)



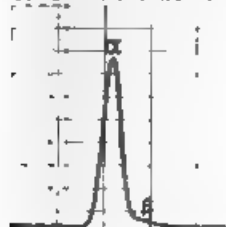
Rt (min): 11.3 12.4  
Derivative: Di-TMS

GURNEA PIG (0-45 fraction)



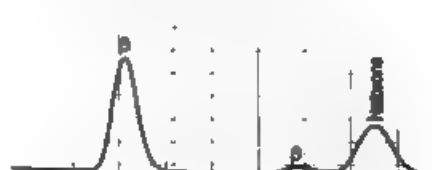
Rt (min): 11.3 12.4 16.7 20.8  
Derivative: Di-TMS Mono-TMS

GURNEA PIG (45-65 fraction)



Rt (min): 11.3 12.4  
Derivative: Di-TMS

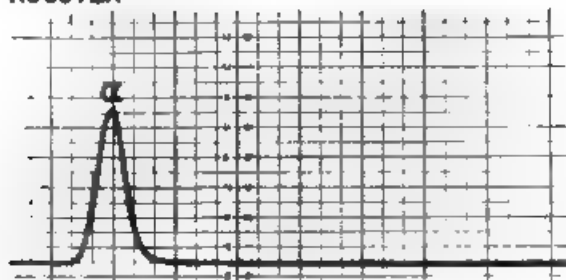
RAT (45-65 fraction)



Rt (min): 12.4 16.1 20.8  
Derivative: Di-TMS Mono-TMS

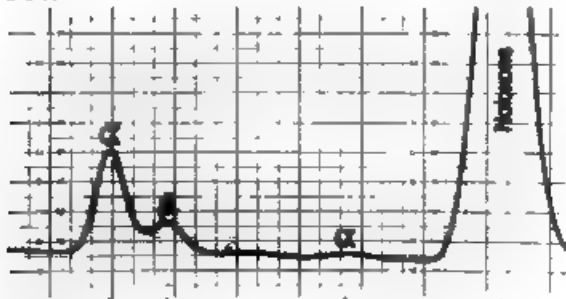
### Naloxone Metabolites

ROOSTER



Rt (min): 11.3  
Derivative: Di-TMS

DUCK

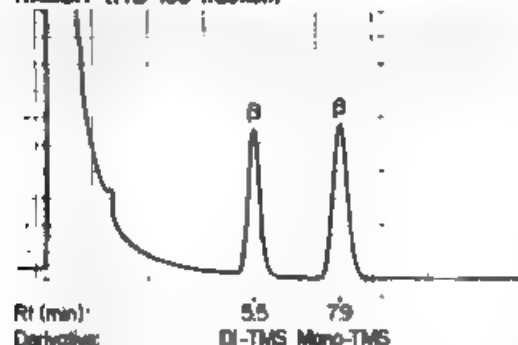


Rt (min): 11.3 12.4 16.7 20.8  
Derivative: Di-TMS Mono-TMS

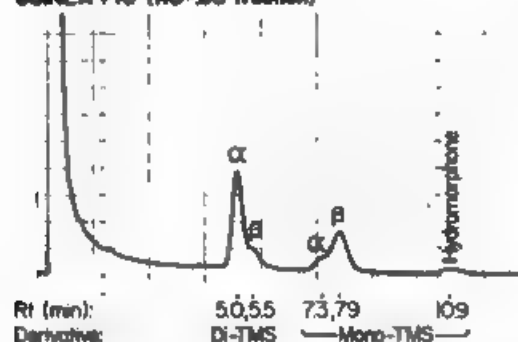
Fig. 6. Display chromatograms at original chart speed of 2cm/min.

**Hydromorphone Metabolites**

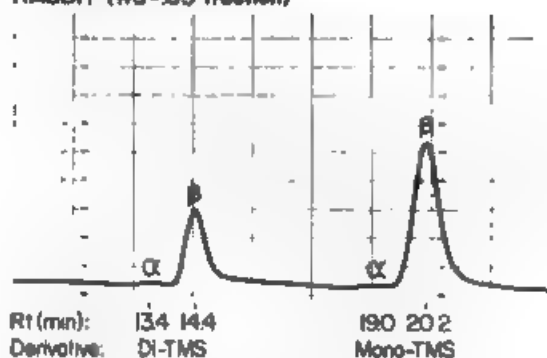
RABBIT (45-65 fraction)



GUINEA PIG (45-65 fraction)

**Naltrexone Metabolites**

RABBIT (45-65 fraction)



GUINEA PIG (45-65 fraction)

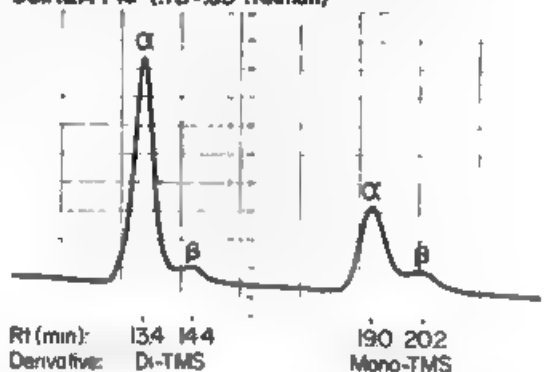


Fig. 7. Display chromatograms at original chart speed of 2cm/min.

from six neonate rabbits (less than one day old) completely reduced naloxone to about 5% of the 6 $\alpha$ -hydroxy epimer and 95% of the 6 $\beta$ -hydroxy epimer. The 45-65% fraction of guinea pig (Harther strain, 3 M) DMKR's completely reduced naloxone to predominantly the 6 $\alpha$ -hydroxy epimer (95%) whereas the 0-45% fraction from the same guinea pig source reduced naloxone 77% to almost an equal quantity of 6 $\alpha$  and 6 $\beta$ -hydroxy diastereomers ( $\alpha$ : $\beta$  = 49:51). The 45-65% fractions of two cat livers assayed independently were without DMKR activity. A 0-45% fraction of one feline liver was assayed and yielded about 1% naloxone reduction to a metabolite which chromatographed as 6 $\alpha$ -naloxol.

Hydromorphone was reduced by chicken (2 M) DMKR and pigeon (1 M and 1 F) to dihydromorphone (6 $\alpha$ -hydroxy), whereas it was reduced by muscovy duck (1 M) DMKR preparation to 6 $\alpha$  and 6 $\beta$ -hydroxy diastereomers. A 0-45% fraction of feline hepatic preparation was inactive toward hydromorphone but reduced about 5% naltrexone to a metabolite which chromatographed as 6 $\alpha$ -naltrexol. A 45-65% fraction of cat liver was ineffective in reducing either substrate. Hydro-

TABLE 6

*Species Stereospecificity Survey with Naloxone*

<u>Order</u>	<u>Genus and species Common name (number)</u>	<u>Configuration</u>		<u>Naloxone Reduction %</u>
		<u>%<math>\alpha</math></u>	<u>%<math>\beta</math></u>	
Anseriformes	<i>Carrina moschata</i> muscovy duck (1)	76	24	26
Columbiformes	<i>Columba livia</i> pigeons (2)	100	0	12
Galliformes	<i>Gallus gallus</i> chickens (4)	100	0	100
Artiodactyla	<i>Bos taurus</i> calf (1)	5	95	56
	<i>Sus scrofa</i> swine (1)	0	100	38
Carnivora	<i>Felis catus</i> cat (2)	—	—	0
Lagomorpha	<i>Oryctolagus cuniculus</i> rabbit (4)	11	89	100
Primata	<i>Macaca rhesus</i> rhesus monkey (1)	14	86	41
Rodentia	<i>Cavia porcellus</i> guinea pigs (3)	95	5	100
	<i>Rattus norvegicus</i> white rats (2)	0	100	78

The 45-70%  $(\text{NH}_4)_2\text{SO}_4$  salted-out DMKR fraction of avian livers and the 45-65% fraction of mammalian livers were employed to reduce naloxone. The chicken and rabbit liver assays were performed twice, once with preparations from two males and once with preparations from two females. The two cat livers were assayed independently. Other assays were performed once.

morphone and naltrexone were reduced by guinea pig DMKR equivalent amount of 6 $\alpha$ -hydroxy and 6 $\beta$ -hydroxy diastereomers produced by the 0-45% fraction and a marked predominance of the 6 $\alpha$ -hydroxy epimer formed by the 45-65% fraction. In contrast, a 45-65% fraction of DMKR's from male rabbit completely reduced hydromorphone to only the 6 $\beta$ -hydroxy carbinol, i.e. dihydroisomorphine.

A stereospecific comparison of hydromorphone, naloxone, and naltrexone reduction using a DMKR preparation from female rabbit revealed a significant difference between the three substrates (Table 7).

In a separate assay using the DMKR preparation from female rabbit liver, oxymorphone was completely reduced to yield approximately 5% 14-hydroxydihydromorphone (6 $\alpha$ -hydroxy) and 95% 14-hydroxydihydroisomorphine (6 $\beta$ -hydroxy). Using a 45-65% fraction of DMKR's prepared from two male rabbit livers, additional substrates were simultaneously reduced. Nalmexone (EN-1620) was reduced to 6-carbinols, 10% of which represented the 6 $\alpha$ -hydroxy epimer and 90% of which represented the 6 $\beta$ -hydroxy epimer. EN-1655 was completely reduced to only the 6 $\beta$ -hydroxy carbinol (isonalbuphine). Oxymorphone was completely reduced with this preparation to about 5% of the 6 $\alpha$ -hydroxy epimer and 95% of the 6 $\beta$ -hydroxy epimer. Hydrocodone, in

TABLE 7

## A Stereospecific Comparison of Substrates

		Hydromorphone	Naltrexone	Naloxone
% $\alpha$ - hydroxy product formed		0, 0, 0	1.8, 0, 2.4	9.7, 9.4, 13.5
Mean		0.00**	1.40*	10.87**
Variance		0.00	1.56	5.22
Standard Deviation		0.00	0.72	1.32
Number		3	3	3
ANOVA F Ratio	= 46.36	Treatment df = 2	Error df = 6	p < .001
*	p < .01	(planned comparison $\psi$ )		
**	p < .001	(planned comparison $\psi$ )		

The three substrates were each completely reduced in triplicate in simultaneous assays using a dialyzed 45-65% ammonium sulfate salted-out fraction of DMKR's prepared from two female rabbit livers. Statistical analysis was by analysis of variance and planned comparison (40).

# Oxycodone Metabolites

## RABBIT (.45-.65 fraction)

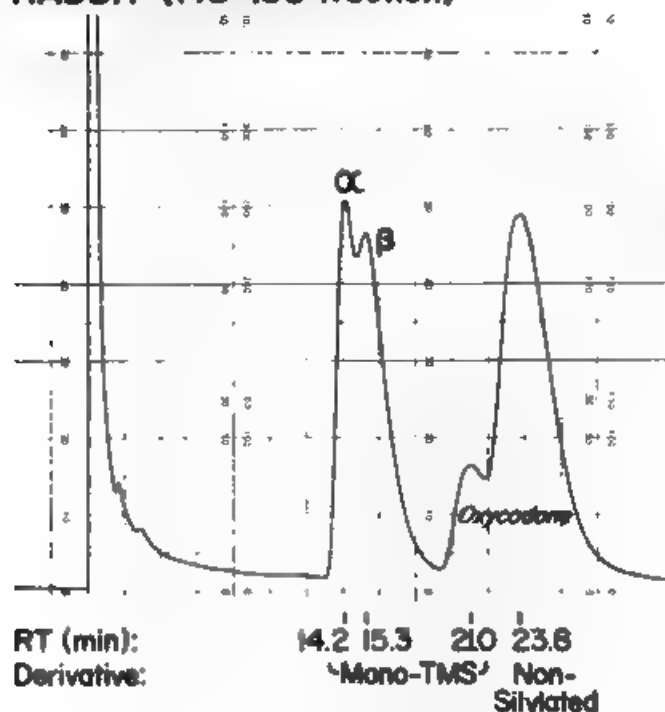


Fig. 8. Chart speed originally at 1/2 cm/min.

contrast, was approximately 29% reduced to only dihydrocodeine (6 $\alpha$ -hydroxy), whereas oxycodone was about 60% reduced to 6-carbinols, 52% of which was 14-hydroxydihydrocodeine (6 $\alpha$ -hydroxy) and 48% of which was 14-hydroxydihydroisocodeine (6 $\beta$ -hydroxy) (Figure 8). Both hydrocodone and oxycodone were completely reduced with a 45-70% ammonium sulfate salted-out fraction from two rooster livers to 6 $\alpha$ -hydroxy carbinols.

No 6-carbinols were detected upon GLC analysis of standard substrates. Standard EN-2265 (6 $\alpha$ -naloxol), EN-2260 (6 $\alpha$ -naltrexol), 6 $\beta$ -naltrexol, dihydromorphine, and dihydrocodeine were pure according to GLC. A batch of 6 $\alpha$ -naltrexol from the National Institute on Drug Abuse contained about 6% 6 $\beta$ -naltrexol, EN-2261 K (6 $\alpha$ -nalmexol) contained about 8% 6 $\beta$ -nalmexol, and nalbuphine contained about 4% isonalbuphine. EN-2370 (14-hydroxydihydromorphine) contained about 12% of the 6 $\beta$ -hydroxy epimer.

Mass spectra of the trimethylsilyl derivatives contained a base peak at  $m/e$  73 corresponding to a TMS fragment. When limited mass spectra were obtained for  $m/e$  greater than 100, bis-TMS-dihydromorphine and the metabolite identified as dihydroisomorphine displayed the same parent and base peak at  $m/e$  431 (Figure 9). The limited mass spectra of bis-TMS derivatives of 14-hydroxydihydromorphine and N-substituted analogs such as 6 $\alpha$ -naloxol contained a base peak at  $M^+-15$

corresponding to loss of a methyl group from the parent peak, whereas for bis-TMS derivatives of 14-hydroxydihydroisomorphine and analogs such as 6 $\beta$ -naloxol the base and parent peaks coincided.

### Observations

Mixtures of C-6 epimers containing about 10-15% of the 6 $\beta$ -hydroxy diastereomers have been detected after sodium borohydride reduction of naloxone and naltrexone (41,42), whereas 6-keto reduction of these 14 $\beta$ -hydroxy dihydronor-morphinone derivatives with lithium tri-*sec*-butylborohydride has been found to be stereospecific for the 6 $\alpha$ -hydroxy epimers (42). In contrast, 6-keto reduction of naloxone and naltrexone with formamidinesulfinic acid has been shown to be stereospecific for the 6 $\beta$ -hydroxy reduction products (43). On the basis of the stereospecificity observed in the enzymatic 6-keto reduction of dihydromorphinones and dihydrocodeinones, one could propose that a limited number of enzymes have a multiplicity of stereospecificities toward these substrates. It is generally regarded in enzymology, however, that different stereospecific activities are the result of catalysis by different stereospecific enzymes (44,45,46,47). This concept is particularly evident with respect to enzymes involved in biotransformation of steroids (48,49). Pollock (38) thus recognized that there are a minimum of at least five different types of enzymes which reduce the 6-ketone moiety of dihydromorphinones and dihydrocodeinones.

DMKR of chicken (Type I DMKR) produces 6 $\alpha$ -hydroxy reduction products from dihydromorphinone and 14-hydroxydihydromorphinones whereas a DMKR of rabbit (Type II DMKR) produces 6 $\beta$ -hydroxy metabolites from these substrates. These may be regarded as hydromorphone keto reductases. Rabbit also possesses an oxymorphone keto reductase (Type III DMKR) which produces 6 $\alpha$ -hydroxy metabolites from 14-hydroxydihydromorphinones. Although pigeon possesses a Type I DMKR, muscovy duck has enzymes with Type I and Type II DMKR activity. Mammalian DMKR's are predominantly of Type II and to a lesser extent probably of Type III. Guinea pig, however, displays mostly Type I DMKR activity *in vitro* yielding a predominance of 6 $\alpha$ -hydroxy carbinols.

Although chicken hens have been shown to reduce naloxone *in vivo* to 6 $\alpha$ -naloxol (19), guinea pigs reduce naltrexone *in vivo* almost exclusively (at least 97%) to 6 $\beta$ -naltrexol (10,42). Thus it appears that in the guinea pig, Type II DMKR is the most important *in vivo*. The domestic cat is anomalous not only in that it conjugates naloxone principally with sulfuric acid rather than glucuronic acid (50) but also in that, for all practical purposes, it lacks DMKR activity.

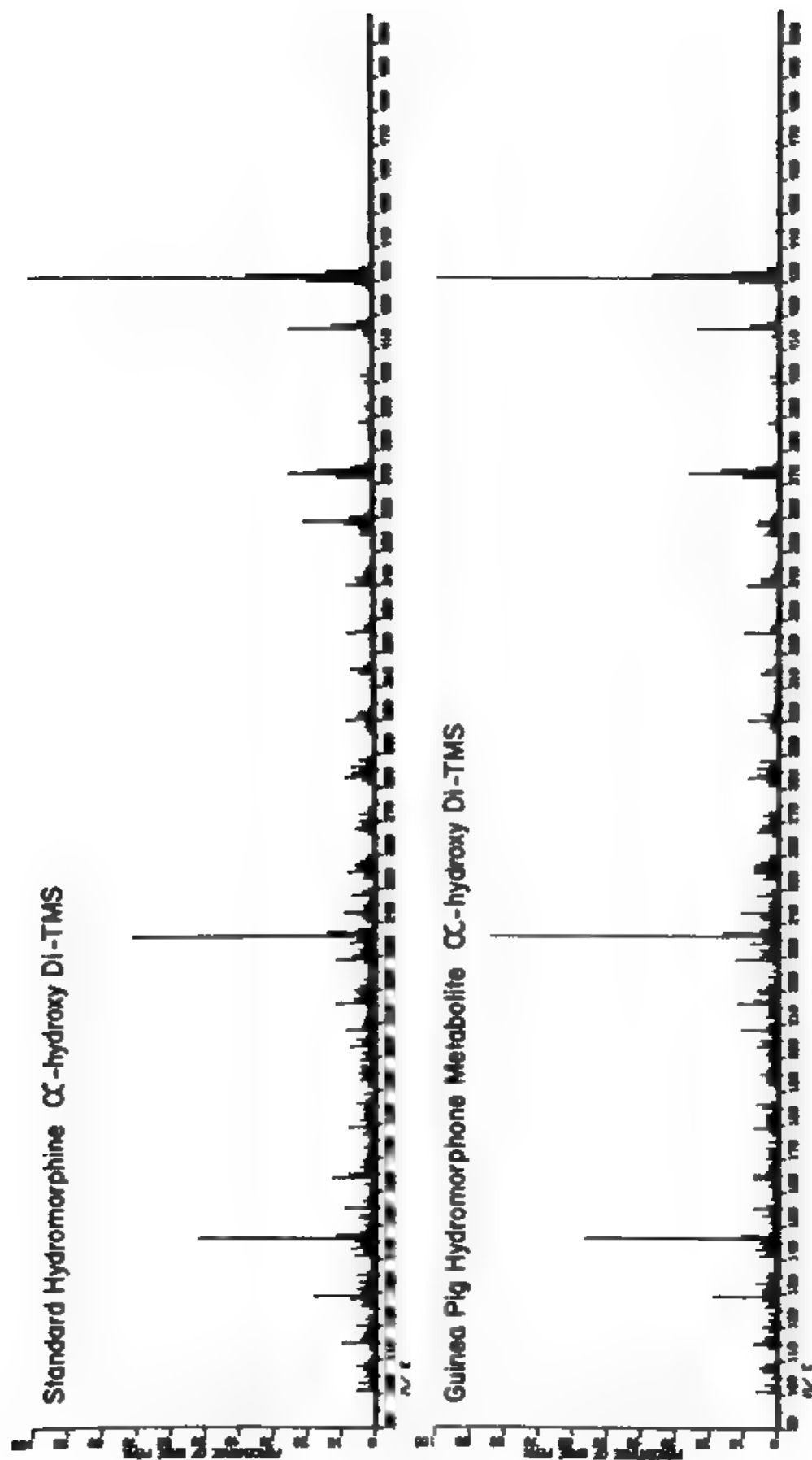


Fig. 9. Limited Mass Spectra of Dihydromorphone and Dihydroisomorphine

Guinea Pig Hydromorphone Metabolite  $\beta$ -hydroxy Di-TMS

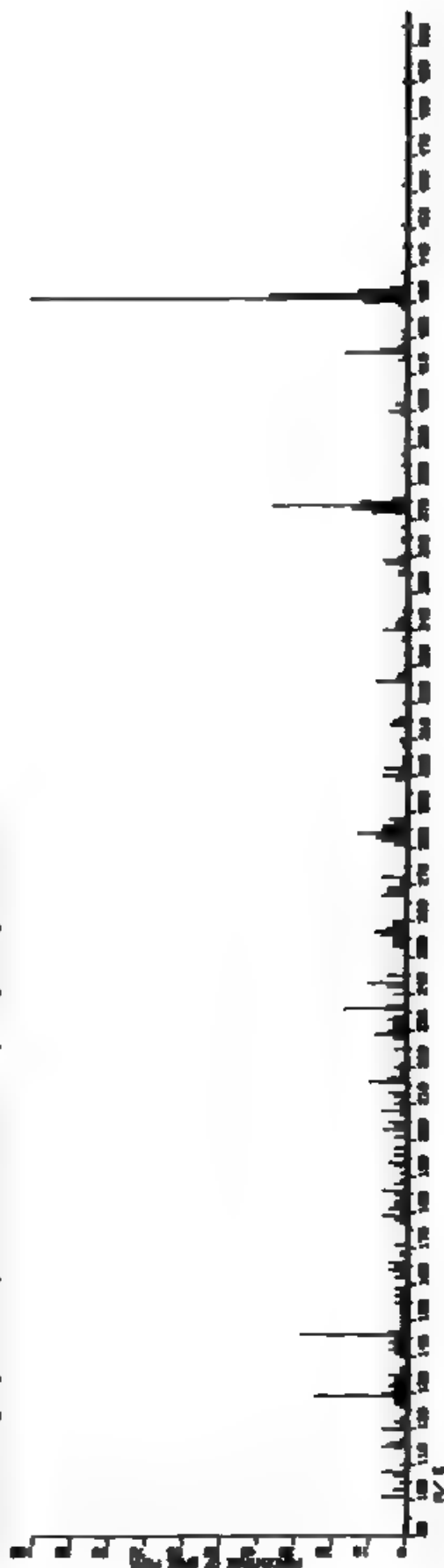


Fig. 9. Limited Mass Spectra of Dihydromorphine and Dihydroisomorphine (continued)



Since with rooster hepatic preparation hydrocodone and oxycodone are reduced to 6 $\alpha$ -hydroxy carbinols as is hydromorphone, the dihydrocodeinone ketone reductase (DCKR) activity in that species may be due to Type I DMKR. Since with rabbit hepatic preparations, however, hydromorphone is reduced to the 6 $\beta$ -hydroxy carbinol whereas hydrocodone is reduced to the 6 $\alpha$ -hydroxy carbinol, rabbit hydrocodone keto reductase (Type I DCKR) is a separate enzyme. The reduction of oxycodone by rabbit hepatic preparation at about twice the rate for hydrocodone and to roughly equal proportions of 6 $\alpha$  and 6 $\beta$ -hydroxy diastereomers indicates there is also a rabbit oxycodone keto reductase (Type II DCKR). Whereas hydrocodone keto reductase reduces hydrocodone and oxycodone to 6 $\alpha$ -hydroxy carbinols, oxycodone keto reductase reduces oxycodone but not hydrocodone to a 6 $\beta$ -hydroxy carbinol.

Since metabolic reactions are theoretically reversible, dihydromorphinone and dihydrocodeinone ketone reductases should be designated as oxido-reductases. Type I DMKR, of which the chicken hepatic enzyme is the prototype, is dihydromorphine: NADP<sup>+</sup> oxidoreductase and Type II DMKR, of which the rabbit hepatic enzyme is the prototype, is dihydroisomorphine: NADP<sup>+</sup> oxidoreductase (22,23). Type III DMKR, of which the rabbit hepatic enzyme may be considered the prototype, is a 14-hydroxydihydromorphine: NADP<sup>+</sup> oxidoreductase. Type I DCKR (Type IV DMKR), of which the rabbit hepatic enzyme may be considered the prototype, is a dihydrocodeine: NADP<sup>+</sup> oxidoreductase. Type II DCKR (Type V DMKR), of which the rabbit hepatic enzyme is also the prototype, is a 14-hydroxydihydroisocodeine: NADP<sup>+</sup> oxidoreductase. An alternative designation for Type I DCKR is 3-O-methyldihydromorphine: NADP<sup>+</sup> oxidoreductase, whereas Type II DCKR may be regarded as a 3-O-methyl-14-hydroxydihydroisomorphine: NADP<sup>+</sup> oxidoreductase.

Although naloxone, naltrexone, and hydromorphone were found to be reduced at similar rates by rabbit hepatic DMKR preparation using a sensitive radioassay (22,23), hydromorphone and naltrexone have now been found to yield significantly more of the 6 $\beta$ -hydroxy reduction product *in vitro* than does naloxone, whereas naloxone yields significantly more of the 6 $\alpha$ -hydroxy reduction product. These findings suggest differences in affinity of these compounds toward Type II and Type III DMKR's from rabbit. Since only isonalbuphine (6 $\beta$ -hydroxy diastereomer of nalbuphine) has been detected after reduction of EN-1655 and this substrate appeared to have significantly greater affinity for rabbit hepatic Type II DMKR by indirect radioassay (22,23), EN-1655 seems to be the best substrate tested for rabbit Type II DMKR. Oxymorphone seems to be intermediate between naltrexone and naloxone in the  $\alpha$ : $\beta$  ratio of 6-carbinols produced by rabbit DMKR's. Thus the relative

order of affinity of these dihydromorphinones toward the prototype II DMKR would appear to be EN-1655, hydromorphone, naltrexone, oxymorphone and naloxone. On the other hand, the order of affinity of some of these substrates toward the prototype III DMKR of rabbit would appear to be naloxone, oxymorphone, and naltrexone. Hydromorphone, naloxone, naltrexone, oxymorphone, and EN-1655 are probably similar as substrates for Type I DMKR's.

The substrate receptors of DMKR's do not seem to be related to CNS opiate receptors. While the antagonist naloxone and agonist oxymorphone are quite similar as substrates in the DMKR system, these compounds display major differences in CNS opiate receptor binding after treatment with the protein modifying reagent iodoacetamide (51) (Table 8).

TABLE 8

*Effect of iodoacetamide on receptor binding of  $^3\text{H}$ -opiate agonists and antagonists*

Opiate	Stereospecific opiate binding (c.p.m)		
	Iodoacetamide		
	Control	Treated	% Change
<b>Antagonists</b>			
$^3\text{H}$ -naloxone	1,040	1,092	+ 5
$^3\text{H}$ -levallorphan	1,551	1,672	+ 8
<b>Agonists</b>			
$^3\text{H}$ -oxymorphone	719	401	-44
$^3\text{H}$ -levorphanol	1,288	827	-36
$^3\text{H}$ -dihydromorphine	1,871	878	-53

*Rat brains were homogenised in 20 volumes of standard Tris buffer and centrifuged at 50,000g for 15 min. The pellet was resuspended in 100 volumes of standard Tris buffer and equal volumes were incubated in the presence and absence of 20mM iodoacetamide for 20 min at 25° C. The homogenates were then centrifuged as before, resuspended in their original volumes and assayed with either (+)-3-hydroxy-N allylmorphinan or (-)-3-hydroxy-N allylmorphinan(levallorphan) at 200nM and the appropriate  $^3\text{H}$ -opiate. Samples were filtered and counted. The following concentrations of  $^3\text{H}$ -opiates were used 1.7nM  $^3\text{H}$ -naloxone, 2.8 nM  $^3\text{H}$ -levallorphan, 3.7nM  $^3\text{H}$ -oxymorphone, 4nM  $^3\text{H}$ -levorphanol, and 0.7nM  $^3\text{H}$ -dihydromorphine.*

[Data from Wilson et al (51).]

DMKR's and DCKR's presumably are involved in some aspect of intermediary metabolism, but their possible conspecificity with other enzymes and normal biological roles remain to be clarified. DMKR's and DCKR's nevertheless are active in xenobiosis, functioning in a capacity of detoxification as evidenced by structure-activity relationships between dihydromorphinones as well as dihydrocodeinones and their 6-keto reduction products.

Potent narcotic analgesic activity is associated with a

6-ketone moiety. Hydromorphone (7,8-dihydromorphinone) has ten times the analgetic potency of morphine in man (52), whereas the narcotic agonist activity of dihydromorphone seems to be about three times that of morphine (53,54). Dihydroisomorphine (6-iso-7,8-dihydromorphone or "dihydro- $\alpha$ -isomorphine" according to nomenclature by some authors) is weaker than dihydromorphone, being very similar to morphine in its potency (55,56). Dihydrocodeinone (3-O-methyl-dihydromorphinone) is also similar to morphine in potency (53,57), whereas dihydrocodeine seems to be at least six times less potent as an antinociceptive agent (53,58). Dihydroisocodeine appears to be closer to morphine in analgetic potency (56,59). While oxymorphone (14-hydroxydihydromorphinone) is ten times more potent than morphine in man (58) and oxycodone (14-hydroxy-dihydrocodeinone) appears to have about four times the antinociceptive potency of morphine as determined by the phenylquinone writhing test in mice (60), their 6-keto reduction products would be expected to have weaker agonist activity.

Potent narcotic antagonist activity is associated with appropriate N-substitution, a 6-keto group and a 14-hydroxyl moiety (61). N-allyl-7,8-dihydronormorphinone has been estimated to be about 1.3 times more potent than nalorphine (N-allylnormorphine) as a narcotic antagonist (62). As measured by the rat tailflick response, N-allyldihydronormorphinone lacked antinociceptive activity whereas nalorphine had less than 0.1 the analgesic activity of morphine (62). N-allyldihydronormorphine was found to have 0.7 the antagonist potency of nalorphine and was also weaker than nalorphine in agonist activity (62). Naloxone (N-allyl-14-hydroxydihydronormorphinone) is between ten to nineteen times more potent than nalorphine as a narcotic antagonist (63,64). While nalorphine is equianalgetic to morphine in man (65,66), naloxone at most has very minimal antinociceptive activity (63,64,67). Naltrexone (N-cyclopropylmethyl-14-hydroxydihydronormorphinone) has been determined to have up to thirty-nine times the potency of nalorphine as a narcotic antagonist and possesses very minimal agonist activity (63,64). In man naltrexone is longer acting than naloxone and is about seventeen times more potent than nalorphine as an antagonist (68). The 6-keto reduction products of naloxone and naltrexone display a substantial decrease in narcotic antagonist activity. EN-2265 (6 $\alpha$ -naloxol) is only about twice as potent as nalorphine as an antagonist (69) and 6 $\beta$ -naloxol is also a weak antagonist (21,43). EN-2260 (6 $\alpha$ -naltrexol) is similar in potency to 6 $\beta$ -naloxol as a narcotic antagonist and 6 $\beta$ -naltrexol is even weaker. Whereas 6 $\alpha$ -naloxol has been found to possess up to about one-fifth the analgesic potency of morphine (69) and 6 $\alpha$ -naltrexol also possesses significant analgesic activity, their 6 $\beta$ -hydroxy epimers are practically devoid of

antinociceptive activity (43). EN-1655 (N-cyclobutylmethyl-14-hydroxydihydronormorphinone) has about one-third the potency of naloxone as a narcotic antagonist but has antinociceptive potency in the morphine range (63,64). Nalbuphine (6 $\alpha$ -hydroxy reduction product of EN-1655) also is very similar to morphine in analgesic potency but has only about one-fourth the potency of nalorphine as an opiate antagonist (70). Nalmexone (N-3',3'-dimethylallyl-14-hydroxydihydronormorphinone) is a weak narcotic antagonist possessing roughly one-half the potency of nalorphine and is about one-third as active as morphine in antinociception (63,64).

Despite the low narcotic antagonist potency of 6 $\beta$ -naltrexol, there is now some evidence that this metabolite contributes to the relatively long duration of narcotic antagonist action of naltrexone in man (71,72). On the other hand, naloxone has been found to demonstrate narcotic agonist activity in the pigeon as determined by key pecking activity in response to stimulus presentations (73,74,75) and 6 $\alpha$ -naloxol also possesses such activity (73). Although naloxone also shows some agonist activity in behavioral experiments in monkeys (74,75), naloxols would not be expected to be generated in sufficient quantity in such mammals to make a significant contribution toward that activity.

#### FURTHER STEREOSPECIFIC STUDIES IN VITRO

After the discovery of enzymes with Types III, IV, and V DMKR activity, an investigation was undertaken to obtain further information on species stereospecificity in the metabolism of dihydromorphinones and dihydrocodeinones. Since Pollock and Dear (37) obtained only 6 $\beta$ -naloxol when naloxone was reduced using a dialyzed salted-out rat hepatic cytosol preparation whereas Misra et al. (3) reported a predominance of 6 $\alpha$ -naloxol and some 6 $\beta$ -naloxol produced by a more crude hepatic preparation of rat, the methodology was modified to directly employ 10 ml of hepatic supernatant prepared by ultracentrifugation at  $R_{ave} = 130,300 \times g$  ( $R_{max} = 176,000 \times g$ ) and the endogenous NADPH generating systems were supplemented with 120  $\mu$ mole glucose-6-phosphate, 7.5  $\mu$ mole NADP<sup>+</sup> and 30 units crystalline glucose-6-phosphate dehydrogenase. GLC analysis was accomplished on a 1.0m x 3mm i.d. glass column packed with 3% OV-17 on 100/120 mesh using a Shimadzu Gas Chromatograph GC-5A with an FID. The column temperature was at 210°C while the injector and detector were at 290°C. Air and hydrogen flows were at 0.9 L/min and 35 ml/min respectively and N<sub>2</sub> flow was at 60 ml/min. GLC-MS was obtained for confirmation of metabolite identification.

A reference batch of 14-hydroxydihydroisocodeine was prepared by formamidinesulfinic acid reduction of oxycodone

with a slight modification of conditions used by Chatterjie et al. (43) for preparation of 6 $\beta$ -hydroxy reduction products of naloxone and naltrexone. A quantity of 2 mmole oxycodone $\cdot$ HCL was reduced in a 500 ml Ehrlemeyer flask employing 16 mmole formamidinesulfinic acid in 130 ml aqueous solution basified to pH 12.0 with 2.22g NaOH. The reaction was allowed to proceed under a current of N<sub>2</sub> for ninety minutes on a hot plate at 85°C employing a magnetic stirrer. The remaining oxycodone (10%) and reduction product (90%) were then extracted into 200 ml ethyl acetate and evaporated to dryness. Analysis by GLC and confirmation by GLC-MS indicated stereospecific reduction to 14-hydroxydihydroisocodeine.

Results of stereospecific comparison of hydromorphone, naloxone, hydrocodone, and oxycodone as substrates for DMKR enzyme systems are summarized in Table 9.

In species such as pigeon, muscovy duck, and guinea pig it is possible that both dihydromorphinones and dihydrocodeinones are reduced by Type I DMKR as previously considered for the chicken DMKR system. Further studies may reveal that these species also possess Type IV DMKR (Type I DCKR). The data clearly suggests, however, that species such as rat and hamster are like rabbit in that they possess distinct enzymes with Type II and Type IV DMKR activity.

TABLE 9

*Configuration of C-6 Reduction Products in vitro*

	<u>Hydromorphone</u>	<u>Naloxone</u>	<u>Hydrocodone</u>	<u>Oxycodone</u>
Chicken	$\alpha$	$\alpha$	$\alpha$	$\alpha$
Pigeon	$\alpha$	$\alpha$	$\alpha$	$\alpha$
Muscovy duck	$\alpha,\beta$	$\alpha,\beta$	$\alpha$	$\alpha$
Guinea pig	$\alpha,\beta$	$\alpha,\beta$	$\alpha$	$\alpha,\beta$
Rabbit	$\beta$	$\alpha,\beta$	$\alpha$	$\alpha,\beta$
Rat	$\beta$	$\alpha,\beta$	$\alpha$	$\alpha$
Hamster	$\beta$	$\alpha,\beta$	$\alpha$	$\alpha$

*Substrates were completely metabolized by hepatic cytosol employing the supplemental NADPH generating system.*

Rabbit, rat, and hamster preparations produced dihydroisomorphine rather than dihydromorphine but roughly 10% 6 $\alpha$ -naloxol and 90% 6 $\beta$ -naloxol. Thus besides rabbit, rat and hamster also appear to possess Type III DMKR activity. Guinea pig like rabbit also possesses Type V DMKR (Type II DCKR) activity (Table 10).

TABLE 10

DMKR Enzyme Type	Configuration of 6-OH Product
I. Dihydromorphine: NADP <sup>+</sup> oxidoreductase (chicken, guinea pig, muscovy duck, pigeon)	$\alpha$
II. Dihydroisomorphine: NADP <sup>+</sup> oxidoreductase (rabbit, rat, hamster, muscovy duck)	$\beta$
III. 14-Hydroxydihydromorphine: NADP <sup>+</sup> oxidoreductase (hamster, rabbit, rat)	$\alpha$
IV. Dihydrocodeine: NADP <sup>+</sup> oxidoreductase (hamster, rabbit, rat, chicken, guinea pig, muscovy duck, pigeon)	$\alpha$
V. 14-Hydroxydihydroisocodeine: NADP <sup>+</sup> oxidoreductase (rabbit, guinea pig)	$\beta$

## STEREOSPECIFIC STUDIES IN VIVO

The isolation of free 6 $\beta$ -naltrexol from human urine (18) stimulated stereochemical studies of narcotic antagonist metabolites from laboratory animals. The *in vivo* naloxone reduction product from chicken hen, for instance, was then determined to be 6 $\alpha$ -naloxol (19), but it was estimated that as much as 5% of the C-6 diastereomer could have been undetected (21). Malspeis et al. (42) reviewed and conducted some *in vivo* studies with naltrexone and determined "that substantially greater quantities of  $\beta$ -naltrexol and/or its conjugates were excreted in the urine of man, monkey, guinea pig and rabbit after administration of naltrexone, whereas very small quantities were excreted by the mouse, rat and dog." They also detected trace amounts of 6 $\alpha$ -naltrexol in the urine of monkey and guinea pig, the latter finding being consistent with that of Cone et al. (20).

*In vitro* studies of naloxone, naltrexone, and hydromorphone metabolism generally have correlated well with *in vivo* metabolic studies. For instance, Pollock and Dear (37) obtained a very sensitive computerized mass spectral scan of metabolites fractionated by GLC which detected no 6 $\beta$ -carbinol produced from naloxone by chicken cock or hen liver. On the other hand, hepatic cytosol of guinea pig liver usually produces a predominance of 6 $\alpha$ -naloxol and 6 $\alpha$ -naltrexol (ca. 95%). The stereospecific differences observed for guinea pig hepatic preparations and *in vivo* metabolism of naltrexone

remain to be reconciled but may well be resolved when the stereospecificity of guinea pig kidney DMKR's are investigated.

During preliminary *in vitro* studies by Pollock (22,23), Roerig et al. (76) reported dihydromorphine-3-glucuronide as an *in vivo* metabolite of dihydromorphine in the rabbit and still suggest it is a major metabolite (36). Since rabbit *in vitro* studies by Pollock and Dear (37) yielded only the 6-iso-diastereomer of dihydromorphine, *in vivo* studies were undertaken to establish the C-6 stereoconfiguration of the dihydromorphine reduction product in rabbit.

Two 4.0 kg male New Zealand white rabbits were administered 60 mg doses of hydromorphone·HCl subcutaneously twice a day for two days. Urine was collected and refrigerated at 4°C every day for seven days. The total urine from each rabbit was then centrifuged and basified to pH 10 with KOH pellets and  $\text{KHCO}_3$  powder. Metabolites were extracted from basified urine into twice a volume of ethyl acetate and were concentrated by evaporation.

The residual urine was acid hydrolyzed and treated in the same manner as described for unhydrolyzed urine. Analysis by GLC and confirmation by GLC-MS revealed the biotransformation of dihydromorphine to dihydro-6-isomorphine rather than dihydromorphine and most of the dihydroisomorphine occurred as the free metabolite. Naltrexone was reduced in a 4.0 kg male rabbit primarily to 6 $\beta$ -naltrexol, although about 1% 6 $\alpha$ -naltrexol is formed and both 6 $\alpha$  and 6 $\beta$ -OH reduction products were obtained from oxycodone as lagomorph urinary metabolites *in vivo*. Since Roerig et al. (36,76) have neither undertaken a stereochemical investigation of dihydromorphine metabolism nor considered the findings of their own collaborators (19,21) with respect to stereospecific metabolism of the dihydromorphine derivatives naloxone and naltrexone, we must assume their glucuronide metabolite was actually dihydroisomorphine.

From the initial human work of Cone (18) and comprehensive stereospecific studies of naltrexone reduction by Malspeis et al. (42), the contention by Chatterjie et al. (36) that the predominant 6-carbinol produced from naloxone in man is N-allyl-14-hydroxy-7,8-dihydronormorphine rather than the 6 $\beta$ -hydroxy diastereomer should be disregarded. The stereospecific metabolism of naltrexone and hydrocodone was investigated *in vivo* in hamsters. Three male golden Syrian hamsters (weight about 135 g each) were administered 60 mg/kg naltrexone·HCl i.p. twice a day for three days and another three were given hydrocodone·bitartrate 60 mg/kg i.p. b.i.d. for three days. Urine was collected for a week and analyzed by GLC. Free 6 $\beta$ -naltrexol and dihydrocodeine (6 $\alpha$ -OH) were detected in agreement with findings from *in vitro* studies (77). Oxycodone (60 mg/kg i.p. twice a day for two days) was administered to two male Harther strain guinea pigs (460 g and 475

g) and urine was collected for a week, metabolites being analyzed by GLC. Nearly equivalent amounts of free 6 $\alpha$ -OH and 6 $\beta$ -OH carbinols were detected reflecting *in vitro* Type I and II DCKR activity.

#### OTHER METABOLIC PATHWAYS

A recently discovered human metabolite of naltrexone is 2-hydroxy-3-O-methylnaltrexol (78,79). The aromatic hydroxylation pathway would be expected to be mediated by the microsomal phenolase complex (80) and the 3-O-methylation step by catechol-O-methyltransferase (81). Indeed, morphine, nalorphine, phenazocine and levorphanol have been demonstrated by an *in vitro* rabbit hepatic preparation to undergo aromatic hydroxylation and subsequent O-methylation by catechol-O-methyl transferase (82). Although enantiomeric substrate stereoselectivity in the metabolism of narcotic drugs has been investigated only with respect to N-dealkylation, this type of stereospecificity would be expected to be operative for other opiate metabolic pathways as well. While enantiomeric substrate stereoselectivity should occur in the aromatic hydroxylation, 3-O-methylation and 6-keto reduction steps in the formation of 2-hydroxy-3-O-methylnaltrexol,  $\alpha,\beta$ -diastereomeric product stereoselectivity also would be expected to occur in the 6-keto reduction step for which the stereochemistry of the C-6 alcohol has not yet been reported. Most likely a mixture of diastereomers is produced, but the 6 $\beta$ -hydroxy epimer would be expected to predominate in man.

Another type of product stereoselectivity in opiate metabolism occurs in formation of geometrical isomers, in particular *cis-trans* isomers. An example of such stereospecificity occurs in hydroxylation of the terminal methyl groups of the N-dimethylallyl substituent of pentazocine to form the *cis* and *trans* isomers 1,2,3,4,5,6-hexahydro-8-hydroxy- $\alpha,6$ (eq.),11(ax)-trimethyl-2,6-methano-3-benzazocine-3-*cis*-2-buten-1-ol and 1,2,3,4,5,6-hexahydro-8-hydroxy- $\alpha,6$ (eq.),11(ax)-trimethyl-2,6-methano-3-benzazocine-3-*trans*-2-buten-1-ol. Mouse liver 10,000 x g supernatant produced five to ten times more *trans*-alcohol than *cis*-alcohol but rat liver formed two to three times more *cis* isomer than *trans* isomer (83). Incubation of pentazocine with rhesus monkey hepatic enzyme preparation yielded nearly equivalent amounts of *cis* and *trans* isomers. In addition some *trans*-alcohol was stereoselectively metabolized to the *trans*-carboxylic acid 1,2,3,4,5,6-hexahydro-8-hydroxy- $\alpha,6$ (eq.),11(ax)-trimethyl-2,6-methano-3-benzazocine-3-crotonic acid (83).

This preferential formation of *trans*-metabolites from pentazocine in rhesus monkey was also observed *in vivo* by analysis of enzymatically hydrolyzed urine (83). Both *cis*,



*trans* product stereoselectivity and *cis*, *trans* substrate stereoselectivity are also operative in the human metabolism of pentazocine. Although unaltered pentazocine, the *cis*-alcohol, and the *trans*-carboxylic acid are observed as metabolites from hydrolyzed human urine, some *trans*-alcohol would be expected as an additional metabolite (83) since it is an intermediate in formation of the *trans*-acid.

As a final example of metabolic stereospecificity of opiates, it may be pointed out that Malspeis et al. (42) observed that 6 $\alpha$ -naltrexol but not 6 $\beta$ -naltrexol yielded an unidentified urinary metabolite after *in vivo* metabolism by guinea pigs. This represents  $\alpha,\beta$ -diastereomeric substrate stereoselectivity.

## CONCLUSION

Metabolic reactions are catalyzed by enzymes which tend to be asymmetrical by virtue of their numerous asymmetric centers. Biological economy thus dictates that stereospecificity will probably occur in the metabolism of opiate agonist and antagonist drugs whenever isomers are metabolized or produced as a result of metabolic reactions. Since different species may be expected to possess some differences in amino acid sequences of enzyme proteins, species differences in metabolic stereospecificity would be expected and indeed have been observed for all types of opiate metabolic stereospecificity examined.

Species variable substrate stereoselectivity of enantiomers has been demonstrated for a variety of opiates with respect to N-dealkylation. This enantiomeric stereoselectivity would be expected to occur in other opiate metabolic pathways such as glucuronidation, ethereal sulfate formation, keto reduction, O-methylation, O-dealkylation, and hydroxylation.

Species variable product stereoselectivity for geometrical isomers has been demonstrated with respect to N-alkyl hydroxylation of pentazocine and would be expected to occur in other situations where *cis-trans* isomers may be metabolically produced.

Substrate stereoselectivity of geometrical isomers has been observed in the oxidation of *cis*- and *trans*-alcohols to carboxylic acids as evidenced in the preferential metabolic formation of a *trans*-carboxylic acid after N-alkyl hydroxylation of pentazocine. Future studies may be expected to reveal instances where a species specific preference is displayed for formation of *cis*-carboxylic acids in opiate metabolism.

The most recently studied type of species variable stereospecificity in opiate metabolism is product stereoselectivity for  $\alpha,\beta$ -hydroxy diastereomers as observed in the

6-keto reduction of dihydromorphinones and dihydrocodeinones. Five different types of dihydromorphinone ketone reductase (DMKR) enzyme activities have been demonstrated and other types will probably be found in the future. The stereospecific reductions of dihydromorphinones and dihydrocodeinones have not yet been shown to be reversible. If the reverse reactions were to occur, one would expect diastereomeric substrate stereoselectivity to be demonstrated. For example, the oxidation of dihydromorphine would not be catalyzed by Type II DMKR's and the oxidation of dihydroisomorphine would not be mediated by Type I DMKR's.

It is noteworthy that muzzling of the phenolic 3-hydroxyl moiety of dihydromorphinones as occurs in dihydrocodeinones not only produces a diminution of analgesic potency but causes a significant change in stereospecific metabolism. Although there are significant differences in stereospecificity of opiates toward enzyme active sites and toward analgesic CNS receptor sites, stereochemical factors in the metabolism of narcotic agonist and antagonist drugs undoubtedly have a profound influence on pharmacological activity and certainly warrant further investigation.

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## 22. FUTURE RESEARCH ON OPIOID PEPTIDES (ENDORPHINS): A PREVIEW

Avram Goldstein

Addiction Research Foundation, Palo Alto, California, 94304.

In 1856, Claude Bernard presented his memorable lectures on the South American arrow poison curare. Ushering in the era of modern experimental pharmacology, he localized the unique muscle-paralyzing action of this drug to receptor sites in the neuromuscular junction. It took three-quarters of a century, through the discoveries of Langley, Hunt, Loewi, Dale and others, before it became clear that the curare receptors had an endogenous ligand, and that this ligand was acetylcholine.

The subsequent history of pharmacology teaches that, although the universal proposition is unprovable, it has been frequently the case that drug receptors are really receptors for endogenous ligands, that the efficacy of drugs is an accident of molecular architecture.

Our discovery of opiate receptors limited to membranes of neural tissue and with a very high degree of stereospecificity (1) was followed within two years by the development of a greatly improved methodology (2,3) which permitted detailed characterization of the receptors. A very uneven regional distribution in the central nervous system suggested specific neuropharmacologic functions, such as pain modulation. The appearance of these receptors at the vertebrate stage of evolution further suggested some specific functional role. Thus, it became obvious that there should be an endogenous ligand. Our own search for such a ligand began in 1972, but not until 1974, did we turn our attention to hydrophilic compounds like peptides. Meanwhile, the painstaking and persistent efforts of Hughes led to identification of methionine enkephalin and leucine enkephalin, two pentapeptides obtained from brain (4,5). By 1975, we had discovered a pituitary peptide with typical opioid effect, much larger than the enkephalins. These developments are summarized in another chapter of this book (6).

When a large and a small peptide are known to produce essentially the same biologic effect, it is parsimonious to assume that the large is precursor to the small, and we

argued that interpretation at the Airlie House meeting of the International Narcotic Research Club in 1975 (7). Indeed, glutathione (a tripeptide) is the largest peptide known to be synthesized in the vertebrate organism by a direct enzymic peptide synthetase mechanism, so it was also likely, *a priori*, that the pentapeptide enkephalin was synthesized as part of a larger sequence by the usual ribosomal translation mechanism. When the structure of Met-enkephalin became known, and it was evident that its sequence appeared uniquely as residues 61-65 of the pituitary peptide  $\beta$ -lipotropin ( $\beta$ -LPH), the probability increased greatly that this pituitary peptide of 91 residues was the precursor (prohormone).

Was it possible that pituitary  $\beta$ -LPH was cleaved, to yield an active opioid peptide (Endorphin)?\* In collaboration with C.H. Li we showed that the whole  $\beta$ -LPH molecule was inactive, but that all fragments cleaved at the 60-61 bond had endorphin activity (8). Thus, in the enkephalin sequence (Tyr-Gly-Gly-Phe-Met), Tyr must have a free amino terminus, so that the basic free  $\alpha$ -nitrogen of Tyr can furnish the cationic group required in all known ligands that interact with the opiate receptor. We also showed directly, with a synthetic opioid heptapeptide, that blocking this  $\alpha$ -nitrogen abolished opioid activity (9).

What is the relationship between pituitary and brain endorphin? It seemed possible that the pentapeptide enkephalins of brain were actually derived from pituitary endorphin [e.g.,  $\beta$ -LPH-(61-91)] by successive cleavages, followed by passage from the blood into the brain. Hypophysectomized rats, however, showed no decline in their content of brain opioid peptide activity up to a month after operation in comparison with sham operated controls (10). It follows that brain endorphins, including the enkephalins, are synthesized within the brain. We have now shown that opioid activity extracted from brain is associated with peptides in the 3000-dalton range [the size of  $\beta$ -LPH-(61-91)] or larger, as well as with smaller peptides of the size of enkephalin. It remains uncertain at this time whether the native form that interacts with the opiate receptors is large, or whether the pentapeptide is cleaved *in vivo* and released into synaptic clefts (11).

Future research on opiates will certainly be concerned with the physiologic role of the endorphins. We have now shown that there are several distinct types of endorphin, both in pituitary and in brain. One type is derived from  $\beta$ -lipotropin ( $\beta$ -LPH) and comprises  $\beta$ -LPH-(61-91) and various smaller fragments of the class  $\beta$ -LPH-(61-n), the smallest of

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\* Endorphin is a generic term to describe opioid peptides without designating any particular chemical structure.

which is  $\beta$ -LPH-(61-65) (Met-enkephalin). In brain, we find endorphin activity associated with a peptide in the 3000-dalton range, and like methionine-enkephalin, its activity is reduced by cyanogen bromide. It is possible, therefore, that in the native state enkephalins exist as part of a longer sequence, and it is even possible that they arise as degradation artifacts during the isolation procedure. A smaller endorphin we find in beef brain has an apparent molecular weight of about 1200-1400. Its biologic activity is unaffected by cyanogen bromide treatment (thus, it contains no critically placed Met residue), and unlike the endorphins derived from  $\beta$ -LPH, its activity is greatly reduced by trypsin treatment. It could possibly contain Leu-enkephalin, but its structure must be different in some way from that of  $\beta$ -LPH. Finally, in pituitary, we find that most of the endorphin activity is due to peptides that are not identical to the  $\beta$ -LPH endorphins. These endorphins appear to be more potent than  $\beta$ -LPH-(61-91) or Met-enkephalin, while their sensitivity to cyanogen bromide suggests that they may contain Met-enkephalin.

The existence of different endorphins suggests the possibility that there are different endorphinergic systems in brain, probably interacting with non-identical opiate receptors. Pharmacologic evidence had already suggested the presence of different classes of opiate receptor. Martin (12) proposed three types of receptor, designated  $\mu$ ,  $\kappa$ , and  $\sigma$ , based upon differential effects of various classes of opiate agonists and mixed agonist-antagonists. Hutchinson et al. (13) showed that the receptors in guinea pig myenteric plexus and in mouse vas deferens behaved differently with respect to relative affinities for agonists and antagonists.

The most significant research problem concerning the endorphins is to elucidate their physiologic roles. Surprisingly, naloxone, which blocks the opiate receptors, has little pharmacologic effect of its own. It is possible, though unlikely, that even when this antagonist completely blocks the effects of high doses of exogenous opiates, it is incapable of blocking the actions of endorphin released in very close proximity. More likely, the endorphinergic systems are not tonically active, but function on a standby basis, being called into play only by circumstances we have not yet fully learned to duplicate. Electrical stimulation of the periaqueductal grey produces analgesia, which is partially blocked by naloxone (14). Acupuncture analgesia is also blocked to some degree by naloxone (15). And there is some evidence that naloxone increases the sensitivity of mice to the noxious effect of a hot plate (16). On the other hand, this antagonist failed to alter the threshold of aversive stimulation at which rats escape from foot shock (17). It

seems improbable that a neural system should exist to antagonize acute pain (which, after all, has important survival value), but possibly one function of endorphin is to obtund chronic pain. The definitive experiments have not yet been carried out.

It is noteworthy that opiate analgesia is at least in part a consequence of the profound alteration of effective state produced by opiates, causing indifference to all aversive stimuli, including pain. Respiratory depression, release of growth hormone, and inhibition of release of luteinizing hormone are among the many specific effects of opiates. It may well be that the several endorphins serve as neurohumoral agents in a variety of systems, much as do the well known neurotransmitters like acetylcholine and norepinephrine. It is interesting that the opiate receptors are found in high density, not only in pain pathways, but also in areas of brain (e.g., amygdala) which are not thought to be involved in pain.

It has long been apparent the opiate effects are not uniquely related to any of the known neurotransmitters, although changes in content and turnover of several transmitters are associated with opiate actions and with tolerance and dependence. Rather, the opiate receptors seem to subserve a general inhibitory function in various neuronal pathways. I proposed (18) that administration of an exogenous opiate might result in suppression of synthesis of endogenous opioids, by analogy to the effects of administering other hormones. Negative feedback loops control the output of hormones and neurotransmitters to maintain appropriate stimulation of target cells. Administration of thyroid hormone, for example, shuts down endogenous synthesis and release by the thyroid gland. Thus, prolonged administration of heroin, saturating the opiate (endorphin) receptors, could cause a shutdown of endorphin production. Some aspects of the withdrawal syndrome could, therefore, be due to endorphin deficiency. Since the neurotransmitter changes in opiate tolerance and dependence are reversed rather quickly upon opiate withdrawal, it is possible that the primary abstinence syndrome is caused by neurotransmitter imbalance, while the secondary, prolonged abstinence syndrome (with its disturbance of affective state) could be caused by endorphin deficiency. The possibility should also be entertained that a pre-existing, genetically determined endorphin deficiency could predispose to opiate addiction.

Finally, the fact that opiates are primary reinforcers in operant self-administration paradigms suggest that endorphins may play a central role in the "reward system".

It is provocative that one of the few clear effects of naloxone at low dosage is to disrupt appetitive behavior in rats (19,20). One may also speculate that a common pathway

shared by all drugs of abuse could involve endorphin and the opiate (endorphin) receptors. The recent reports that ethanol induced calcium depletion from brain membranes *in vivo* is blocked by naloxone (21) open a fruitful new line of research on the relationship between ethanol and the opiates--research that could lead to better understanding of multiple drug abuse.

It is on theoretical grounds improbable that endorphins (or congeners of endorphins) will prove to be non-addicting analgesics. Nor is it likely that useful new medications will result from molecular modification of the endorphin structures. On the contrary, if the endorphins had been discovered before opiates were known, the challenge would certainly have been to develop stable and lipophilic *non-peptide* analogues to pass the blood-brain barrier and fit the analgesic endorphin receptors. The outcome of such efforts would undoubtedly have been the development of agents like levorphanol, etorphine, meperidine, and methadone. Future research, however, is likely to lead us to more fundamental understanding of the processes of primary reinforcement, tolerance, and dependence, which underlie opiate addiction and other addictive diseases.

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